FREE ERYTHROCYTE COPRO- AND PROTOPORPHYRIN

A METHODOLOGICAL AND CLINICAL STUDY

BY

LARS WRANNE

ALMQVIST & WIKSELLS BOKTRYCKERI AB
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Introduction

The presence of free porphyrin in the erythrocytes was first established by van den Bergh and Hyman (1928) and the porphyrin was later on identified as protoporphyrin IX by Grotepass (1937), i.e. the same porphyrin as that present in hemoglobin. Watson (1950) reported that the free erythrocyte protoporphyrin was accompanied by small amounts of another porphyrin. This was shortly afterwards isolated and identified as coproporphyrin (Schwartz and Wikoff 1952). Using a recently developed semi-quantitative microchromatographic method, Sturgeon, Chen and Bergren (1958) demonstrated the presence of free uroporphyrin in erythrocytes of normal individuals.

The significance of the free erythrocyte protoporphyrin has been discussed greatly during the years since its discovery. Two of the main factors which appear to influence the amount of protoporphyrin are (1) increased erythropoiesis and (2) factors interfering with the utilization of iron in the synthesis of hemoglobin, and iron deficiency. Both these factors have been found to increase the free erythrocyte protoporphyrin. Watson (1950), studying the free erythrocyte coproporphyrin, found that the concentration of this pigment was closely correlated to the reticulocyte count and considered it to be "a chemical index" of the intensity of blood formation. Finally, Sturgeon, Chen and Bergren (1958), in their investigations of free erythrocyte uroporphyrin, found that this pigment was also correlated to the rate of the blood formation since increased amounts occurred in hemolytic anemias.

The present investigation was started in 1956 to study the physiological behaviour of free erythrocyte copro- and protoporphyrin in healthy infants and children and in patients with various hypochromic anemias. A family investigation prompted by a case of hypochromic anemia with disturbed heme-metabolism has been published elsewhere (Wranne 1959). In 1956, uroporphyrin was not known to be a normal constituent of erythrocytes and thus was not separately searched for. Since earlier methods for the analysis of free erythrocyte porphyrins were found insufficient, it proved necessary to devote the major part of the work to methodological studies. Associated problems, such as the nature of the porphyrins and the porphyrin content of plasma, were also studied. Finally, the effect on the free porphyrins of a sudden change from iron deficiency to a state of sufficient iron supply was studied after starting intravenous iron therapy.

PART ONE

Methodological Study

CHAPTER I

Earlier and present methods

1. Short review on earlier methods of determining free copro- and protoporphyrin in erythrocytes

Extraction and purification of protoporphyrin. Since the first description of free red-cell protoporphyrin by van den Bergh and Hyman (1928), at least seven methods for the quantitative determination of protoporphyrin in erythrocytes have been published. The first, proposed by van den Bergh, Grotepass and Revers (1932), comprised extraction from the red cells with a mixture of ethyl acetate and glacial acetic acid for two hours. After being washed with water, this extract was extracted with 5 % HCl. After one purification:

$$\text{HCl} \xrightarrow{\text{Neutralization}} \text{Ether} \xrightarrow{\text{H}_2\text{O wash}} 5\ \%\ \text{HCl}$$

the protoporphyrin was finally estimated by its fluorescence in ultraviolet.

Several modifications of this procedure have been published; the alterations chiefly concern the time of extraction of the erythrocytes, the concentrations of the hydrochloric acid and the methods for the final estimation of the porphyrin. However, the principles of van den Bergh and coworkers have been essentially unchanged. In Table 1, the different modifications are briefly presented. Most of these modifications include erythrocyte coproporphyrin as well as protoporphyrin, hence the term "erythrocyte porphyrin" would be more appropriate. However, the main part generally consists of protoporphyrin.

The great number of modifications indicates that the ideal conditions for the analysis have been difficult to find. The problems inherent in the estimations of these small quantities of protoporphyrin are chiefly dependent on its instability in strongly acid solutions and when exposed to light. In order to obtain a favorable distribution of the porphyrin between organic solvent and hydrochloric acid, and in order to get reasonable concentrations of porphyrin in the final extracts, a strong mineral acid is necessary. Schumm (1939) and Grinstein and Wintrobe (1948) used 25 % HCl. However, in strong acid solutions, the vinyl side chains of protoporphyrin are readily changed to hydroxyethyl with formation of hematoporphyrin. Other changes may occur as well (Schumm 1927, Paul 1953). The formation of hematoporphyrin is of little significance when only protoporphyrin is estimated; if coproporphyrin is also determined, serious errors may be introduced.

Protoporphyrin is rapidly destroyed when exposed to ultraviolet light (Lageder 1936, Grinstein and Watson 1943). Diffuse

TABLE 1. *Methods for the quantitative determination of free erythrocyte porphyrins.*

	v. d. Bergh, Grotepass and Revers 1932	Schumm 1939	Grinstein and Watson 1943	Grinstein and Wintrobe 1948	Schwartz and Wikoff 1952		Present method	
Volume of erythrocytes	10–20 ml	40 ml	5 ml	1 ml	5 ml		2–4 ml	
First extraction and subsequent washings of the precipitate	EtAc/HAc 3/1 100–150 ml 2 hours	EtAc/HAc 3/1 300 ml 30 min. + 100 ml × 3	EtAc/HAc 3/1 50 ml 2 min. + 15 ml × 3	EtAc/HAc 3/1 10 ml 2 min. + 5 ml × 3	EtAc/HAc 4/1 50 ml several times until colorless extract		EtAc/HAc 4/1 40 ml 2 min. + 10 ml × 3	
Washing	H_2O 3–4 times	H_2O 300 ml × 4	H_2O 3 times	H_2O 7–8 ml × 3	3% NaAc 40–50 ml × 3		3% NaAc 15 ml × 3	
First extraction with acid	5% HCl	25% HCl 2 ml × 7–10	10% HCl 2 ml	10% HCl 2 ml	3 *N* HCl 5–10 ml		5 *N* HCl 4 ml × 4	
			until absence of fluorescence in extract					
Neutralization and additions	NaAc		NaAc + 1 ml glac. HAc	NaAc + 1 ml glac. HAc	NaAc		NaAc	
Second extraction by organic solvent	Ether	Chloroform (evaporation, dissolution in HCl)	Ether equal volumes × 3	Ether 10 ml × 2	Ethyl acetate 50–75 ml × 3		Ethyl acetate 50 ml × 3	
Washing			1% Na_2CO_3, few ml + H_2O × 2	1% Na_2CO_3 2 ml × 1 + H_2O 2 ml × 2	H_2O × 1		H_2O 3 ml × 2	
Second extraction with acid	5% HCl		5% HCl 1.5 ml × 3–4	25% HCl 1.5 + 0.3 ml	*First* 0.1 *N* HCl 3–5 ml "exhaustively". Combined extracts washed with $CHCl_3$ 5 ml × 2, washes added to EtAc	*Second* 3 *N* HCl "exhaustively"	*First* 0.24 *N* HCl 4 ml × 6 Extracts not pooled	*Second* 5 *N* HCl 4 ml × 4 Extracts pooled
Physical estimation in final extracts	Fluorometry	Spectrophotometry (photographical method)	Evelyn colorimeter	Beckman DU 409 mμ	Fluorometry	Evelyn colorimeter	Separate readings of fluorescence in each extract	Spectrophotometry at Soret peak, 380 and 430 mμ
Porphyrins probably present in final extracts	Protoporphyrin + coproporphyrin	Protoporphyrin	Protoporphyrin + coproporphyrin	Protoporphyrin + coproporphyrin	Coproporphyrin	Protoporphyrin	Coproporphyrin (+ traces of protoporphyrin)	Protoporphyrin

daylight is also deleterious. Consequently, the protoporphyrin analysis should be performed quickly and as far as possible in the dark. However, high efficiency in extracting requires perfect separation of the phases. This calls for good light and is time-consuming and therefore increases the risk for protoporphyrin loss.

Methods for the physical estimation of protoporphyrin in the final extracts. Van den Bergh and coworkers (1932), Angeleri and Vigliani (1935), Lageder (1936) and Ward and Mason (1950) estimated the protoporphyrin content by its fluorescence in ultraviolet light. Because of the instability of protoporphyrin when exposed to light, many authors have exchanged the fluorometric analysis for estimations of the light absorption. Seggel (1940) measured the absorption in the visible part of the spectrum, while Schumm (1939) determined the light absorption in the Soret band (near ultraviolet) by the photographic method. The use of a photoelectric colorimeter, which Grinstein and Watson (1943) introduced in this field made the study of the Soret band much easier. Even better measurements could be achieved by a photoelectrical spectrophotometer. This allows a check of the identity of the porphyrin by measuring the wavelength of the maximum of the Soret band.

Extraction and purification of proto- and coproporphyrin. The method described by Grinstein and Watson (1943) was modified by Schwartz and Wikoff (1952) in order to permit the simultaneous analysis of proto- and coproporphyrin. The principles of the modification are shown in Table 1. Since this method will be frequently discussed in the following discussion, a short description will be given here.

At least 5 ml of erythrocytes are extracted with a 4:1 mixture of ethyl acetate and glacial acetic acid. The extraction is repeated several times. The combined extracts are washed three times in a separatory funnel with a 2 % aqueous solution of sodium acetate and then extracted exhaustively with 3 *N* HCl. The combined acid extracts are neutralized to Congo red reaction with sodium acetate and the porphyrins transferred to ethyl acetate. The ethyl acetate solution is washed with water and extracted exhaustively with 0.1 *N* HCl. The combined 0.1 *N* HCl extracts are washed with chloroform in order to remove co-extracted traces of protoporphyrin; the washings are added to the ethyl acetate solution. The latter is finally extracted by 3 *N* HCl several times. The porphyrins contents of the 0.1 *N* HCl extracts (coproporphyrin) are determined in a sensitive fluorometer, whereas the protoporphyrin contents of the 3 *N* HCl extracts are measured in an Evelyn colorimeter.

The method just described was used without especial changes in all earlier quantitative investigations of free erythrocyte proto- and coproporphyrin in humans. However, Proel and May (1952) studying scurvy in monkeys, preferred 0.33 *N* HCl to 0.1 *N* for the extraction of coproporphyrin. Since no thorough examination of the efficiency and reliability of the Schwartz and Wikoff method has been published, it was considered necessary for the purpose of the present investigation to submit the method to a closer study. The results obtained convinced the author that the method of Schwartz and Wikoff required modifications in several respects. In the following, the procedure finally adopted is first described. After that, the details of the methodological study are reported.

2. The author's method

The blood sample is centrifuged at 3000 rpm for 30 minutes and the plasma carefully removed. In a separate sample, a hematocrit determination is made. The erythrocytes, 2 to 4 ml, are transferred to a flatbottomed test tube and thoroughly mixed with 40 ml of a 4 : 1 mixture of EtAc[1] and glacial HAc. The precipitate is finely ground with a glass rod for two minutes and placed on a glass filter. The extract is then filtered with gentle suction. The precipitate is washed three times with 10 ml portions of the mixture; the third wash is almost colourless as a rule. The combined EtAc/HAc extracts are washed three times in a separatory funnel with 15 ml of a 3 % aqueous solution of NaAc. The porphyrins are removed from the EtAc/HAc solution by four extractions with 4 ml 5 *N* HCl. The combined HCl extracts are neutralized with NaAc, using Congo red as indicator; then they are extracted three times with 50 ml EtAc. The combined EtAc solutions are washed twice with 3 ml distilled water, the washings being discarded. Sufficient time should be allowed for the separation of the two phases. Finally, the porphyrins are extracted first with 4 ml portions of 0.24 *N* HCl six times, then with 4 ml portions of 5 *N* HCl four times. The coproporphyrin (and traces of the protoporphyrin) goes into the 0.24 *N* HCl extracts, the fluorescence of each being determined by comparison with a coproporphyrin standard. A sensitive fluorometer is required. The sixth extract is considered to be free from coproporphyrin, and its fluorescence is used as blank. After correction for this blank, the coproporphyrin content in the preceding five extracts is calculated. The protoporphyrin content is determined spectrophotometrically in the pooled 5 *N* HCl extracts. The value is corrected according to the principles of Rimington and Sveinssen (1950) using the coefficient given by With (1955). 5 cm cells are used. The method can also be applied to the determination of porphyrin in 5 ml of plasma.

Recovery experiments, described in Chapter V, revealed that the average recovery of added protoporphyrin was about 80 % and that of coproporphyrin about 75 %. The results were therefore corrected by multiplying with the factors 1.25 (protoporphyrin) and 1.33 (coproporphyrin).

3. Reference substances

Protoporphyrin. Protoporphyrin IX dimethyl ester was prepared according to the method of Grinstein (1947). The preparation was performed on blood from adults as well as on blood from newborn babies. The ester was crystallized from methanol and stored in dry state in the dark. Before use, the ester was hydrolyzed at room temperature overnight by 7 *N* HCl. Since protoporphyrin is not stable in strongly acid solution, water was subsequently added to a final acid concentration of 1 *N*. This protoporphyrin was used within a few days. When it was considered important to get protoporphyrin free from hematoporphyrin and other porphyrins with low HCl-numbers, the protoporphyrin was purified according to the principles of Paul (1953). The protoporphyrin is probably also contaminated by other

[1] EtAc: Ethyl acetate, Merck DAB 6, with a trace of EDTA added. HAc: glacial acetic acid, p.a. NaAc: Sodium acetate, p.a.

degradation products. Since the protoporphyrin was chiefly used for recovery experiments these products were thought to be insignificant. Chromatography as described by Eriksen (1953) gave single spots with R_f values between 0.80–0.83. No differences between protoporphyrin prepared from adult blood and cord blood was found.

Coproporphyrin. Coproporphyrin I and III tetramethyl esters were obtained through the courtesy of dr. med. T. K. With, Svendborg, Denmark. The amounts available were very small and then only some tenths of a milligram were hydrolyzed each time. After hydrolysis for 36 hours in 7 *N* HCl, the solutions were diluted to a final acid concentration of 0.2 *N*. When stored in the dark and in highly diluted solution, coproporphyrin was found to be very stable. This has also been noted by Jope and O'Brien (1945). Chromatography according to Eriksen (1958) revealed single spots when coproporphyrin III was applied ($R_f = 0.54$–0.55), but when coproporphyrin I was chromatographed, a double spot was noted, one intense spot with R_f 0.41, another faint spot with R_f 0.53. It seems possible that the I isomer was slightly contaminated by the III isomer, but the presence of another porphyrin cannot be excluded.—The R_f-values found by the present author differ from those found by Eriksen. However, it was noticed that slight differences in the solvents and in the temperature altered the R_f-value considerably. The necessity of using reference substances when chromatographing porphyrins is thus obvious.

No attempt has been made to calculate the specific extinction coefficients of proto- and coproporphyrin. As With (1959) has pointed out, it must be considered safer to use coefficients calculated by investigators who have prepared the substances themselves and have a great experience of their purification. Therefore the coefficients given by Rimington and Sveinssen (1950) as modified by With (1955) have been used. However, the coefficient for coproporphyrin given by With only applies to coproporphyrin dissolved in 0.1 *N* HCl and since the standard solutions for the fluorometric analyses performed during this investigation contained 0.2 *N* HCl, the coefficient had to be recalculated. By comparing coproporphyrin solutions of the same concentrations in 0.1 and 0.2 *N* HCl, With's coefficient 0.817 rose to 0.90. It was found that the presence of ethyl acetate dissolved in the 0.24 *N* HCl extracts increased the fluorescence by about 12 %. To correct this, the coproporphyrin standard solutions, in which no ethyl acetate was present, were made 12 % more concentrated than designated.

During the fluorometric estimation, the standard coproporphyrin solutions were repeatedly exposed to strong ultraviolet light. Nevertheless, no deterioration was noticed during one day's use, and fresh solutions were used every day.

CHAPTER II

Spectrophotometric and fluorometric determination of copro- and protoporphyrin

1. Spectrophotometric determination

The quantitative determination of proto- and coproporphyrin is carried out conveniently by measurements of the light absorption. Porphyrins dissolved in mineral acids show two bands in the visible and one band in the near ultraviolet region. The latter band is very sharp and distinct and suitable for spectrophotometrical analysis. The position of the band is somewhat dependent on the concentration of the acid solvent; in coproporphyrin, the extinction coefficient shows a similar dependency (Table 2).

Grinstein and Wintrobe (1948) introduced the photoelectrical spectrophotometer in order to determine the porphyrin in the final extracts by measuring the maximal extinction in the Soret band. This method has later been used by many other workers. However, as will be demonstrated below, the estimation of a porphyrin by a single measuring of the maximal extinction is seriously disturbed by even small amounts of impurities absorbing in the near ultraviolet region.

Rimington and Sveinssen (1950) have proposed a method for porphyrin analysis in impure solutions. The method is based on the assumption that the light absorption caused by impurities is constant in the region of the Soret band or at least evenly increasing or decreasing. The extinction maximum (E_{max}) and the extinction at 430 and 380 mμ (E_{430}, E_{380}) are measured and the porphyrin concentration is then calculated by aid of a special equation. This method was originally designed for the determination of uroporphyrin in urine but has been found useful for other purposes as well. With (1955) has discussed its application on proto- and coproporphyrin solutions and proposed the following equations:

Protoporphyrin concentration: (μg/ml, in 3 N HCl) = $1.225 \times (2\ E_{max} - E_{380} - E_{430})$
Coproporphyrin concentration: (μg/ml, in 0.1 N HCl) = $0.817 \times (2\ E_{max} - E_{380} - E_{430})$

Since the hematin absorbs light intensely in the 400 mμ-region, the method given by Rimington and Sveinssen could be expected to give better results than a single measurement of the absorption at the Soret peak. In order to study the advantages obtainable by this method, the following experiment was carried out. Erythrocytes were extracted by an ethyl acetate/acetic acid mixture as usual. The washed extract was repeatedly extracted by 5 N HCl to remove all traces of proto- and coproporphyrin. The deep-coloured residue was added to ethyl acetate, in

TABLE 2. *Absorption maxima in near ultra violet and molar extinction coefficients of some porphyrins.*[1]

	Solvent		
	25% HCl	5% HCl	0.3% HCl
Protoporphyrin	411 mμ $\varepsilon = 278{,}000$	407 mμ	
Coproporphyrin	406 mμ $\varepsilon = 437{,}000$	402 mμ	401 mμ $\varepsilon = 531{,}000$
Hematoporphyrin	407 mμ	403 mμ	

(1) Data from Schumm (1939), Grinstein and Wintrobe (1948) and Jope and O'Brien (1945).

which known amounts of protoporphyrin had been dissolved. The volume of the added matter was 1 : 5 and 1 : 50 of the ethyl acetate volume. The protoporphyrin was extracted by 5 *N* HCl, care being taken to obtain perfect separation between the two phases. The HCl extracts appeared quite clear. Two extractions were carried out; and in the combined extracts, the light absorptions at the Soret band peak, at 430 and at 380 mμ were measured. The protoporphyrin content was calculated from the maximal absorption, using the coefficient given by Grinstein and Wintrobe (1948); it was also calculated from the three readings using the method of Rimington and Sveinssen (1950) as modified by With (1955). The results are found in Table 3 and Fig. 1. In

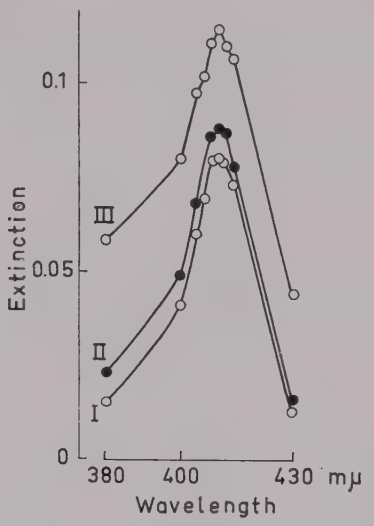

Fig. 1. Influence of impurities on the near ultraviolet spectrum of protoporphyrin solutions. I, II and III refer to the numbers in Table 3.

other experiments, the results of complete analyses of free proto- and coproporphyrin using the Rimington–Sveinssen (1950) correction were compared to the results obtained with a single measuring of the maximal extinction. The analyses were carried out on pure samples of protoporphyrin as well as on samples of erythrocytes. The results are found in Table 4.

Discussion

In the routine analysis of free erythrocyte proto- and coproporphyrin, slight contamination of the final HCl extracts

TABLE 3. *Influence of impurities on the estimation of protoporphyrin in HCl-extracts.*

	Addition of impurity to ethyl acetate	Addition of protoporphyrin μg	Recovery of protoporphyrin μg calculated according to	
			Grinstein & Wintrobe	Rimington & Sveinssen
I	none	1.96	1.80	1.79
II	1:50	1.96	1.98	1.86
III	1:5	1.96	2.59	1.74

TABLE 4. *Complete analysis of free erythrocyte protoporphyrin, results calculated acc. to Grinstein and Wintrobe (1948) and Rimington and Sveinssen (1950).*

Test sample	Protoporphyrin in micrograms, calculated according to	
	Grinstein & Wintrobe	Rimington & Sveinssen
Protoporphyrin, 0.4 μg	0.36	0.28
Protoporphyrin, 0.8 μg	0.54	0.61
Protoporphyrin, 1.2 μg	0.93	0.92
Erythrocytes	0.87	0.39
Erythrocytes	0.74	0.37

by hematin is difficult to avoid despite very careful separation of the ethyl acetate and the HCl phases. The contamination increases light absorption in the near ultraviolet region. If protoporphyrin is estimated by the light absorption maximum in the Soret band in the presence of hematin, the results obtained are too high. The findings shown in Table 3 indicate that successful corrections can be made by aid of a correction proposed by Rimington and Sveinssen (1950) comprising spectrophotometrical readings at 380 and 430 mμ as well.

When the complete procedure for the analysis of free erythrocyte proto- and coproporphyrin was carried out on pure protoporphyrin solutions, it was found that, in the absence of hematin, the results of single measurements of the Soret peak are equal to those obtained by the methods of the authors just mentioned. In contrast to these findings (Table 4), analyses of samples of erythrocytes gave widely different results according to the method of calculating the results. The greater values obtained by calculating the protoporphyrin amount from reading at the Soret peak only are thought to depend on hematin contamination. More reliable results were obtained by using the Rimington–Sveinssen correction. Recovery experiments, described later, indicate that this procedure does not give falsely low protoporphyrin values.

Considering the experimental findings now described, the present author chose to use the Rimington and Sveinssen method (1950) as modified by With (1955) for calculating the amounts of protoporphyrin from spectrophotometrical readings. Previously, this method has only been used by Rubino, Pagliardi, Prato and Giangrandi (1958), by Koskelo (1959) and by Prato, Mazza and Fiorina (1959) for the determination of free erythrocyte protoporphyrin.

2. Fluorometric determination

The fluorescence of coproporphyrin has been studied by i.a. Fikentscher (1932), Jope and O'Brien (1945) and Schwartz, Zieve and Watson (1951). The reports of these authors indicated that fluorometric determination was convenient for very dilute coproporphyrin solutions. The intensity of the fluorescence, however, was dependent on a number of factors; e.g. pH, the presence of ethyl acetate, the

wave-length of the exciting light, etc. During the present investigation, it was found necessary to test the influence of these and some other factors.

In fluorescence estimations, it is also important to choose suitable wave-lengths of the exciting and the emitted light in order to obtain maximum sensitivity and minimum disturbance by scattered light. Since recent studies of the porphyrin fluorescence's activation and emission spectra were lacking, the present investigation was first directed to a spectrofluorometric study of proto- and coproporphyrin.

Fluorescence emission and activation spectra

Apparatus. Emission spectra: Zeiss PMQ II spectrophotometer equipped with the fluorescence accessory ZFM 4 was arranged in the following manner. The light from a mercury lamp, filtered to isolate the 404.7 mμ line, excited the fluorescence. The emitted light passed through the monochromator to the photomultiplier. The slit was set to 1.5 mm. Fluorescence intensity, as judged by the galvanometric deflection, was plotted against wavelength.

Activation spectra: Using the same apparatus, the light from a tungsten lamp, passing through the monochromator, was allowed to excite fluorescence in the sample. The emitted light was filtered through Jena OG 1, 2 mm and registered by a photomultiplier. The slit was set to 1 mm. The amplification was maximal.

When discussing the emission and activation spectra, it must be kept in mind that the galvanometric deflection, which is considered to be a measure of the fluorescence intensity, is subject to a number of additional influences. Emission spectra are influenced by the response of the photomultiplier at different wavelengths and in some degree by the dispersion of the monochromator. Activation spectra are also dependent on the spectral energy distribution of the light source. Corrections for these errors were found to be difficult and uncertain, and the results given below are uncorrected and only valid for this type of instrument.

Results

A study of the *emission spectra* of copro- and protoporphyrin, dissolved in 0.2 *N* HCl revealed that the spectra were essentially equal although the fluorescence intensity of protoporphyrin was weaker than that of coproporphyrin. The highest intensity was found at 590 mμ for coproporphyrin and between 590 and 600 mμ for protoporphyrin (Fig. 2).

A study of the *activation spectra* of proto- and coproporphyrin dissolved in 0.2 *N* HCl and 2 *N* HCl showed, that maximum excitation of fluorescence occurred at sharply defined wavelengths. If a more narrow slit had been used, the peaks would probably have been still sharper, but narrower slits produced too little light-emission to allow safe readings on the galvanometer. A comparison between proto- and coproporphyrin could be made (Figs. 3 and 4). Coproporphyrin exhibited maximum fluorescence at a wavelength distinctively different from that of protoporphyrin. The activation spectra of coproporphyrin I and III were found to be identical, and the III isomer was used throughout the investigation. The wavelength for maximum excitation was found to be approximately the same as the light absorption peak under the same conditions.

Factors influencing fluorescence intensity

Apparatus. The Zeiss spectrophotometer with fluorescence attachment was used. The

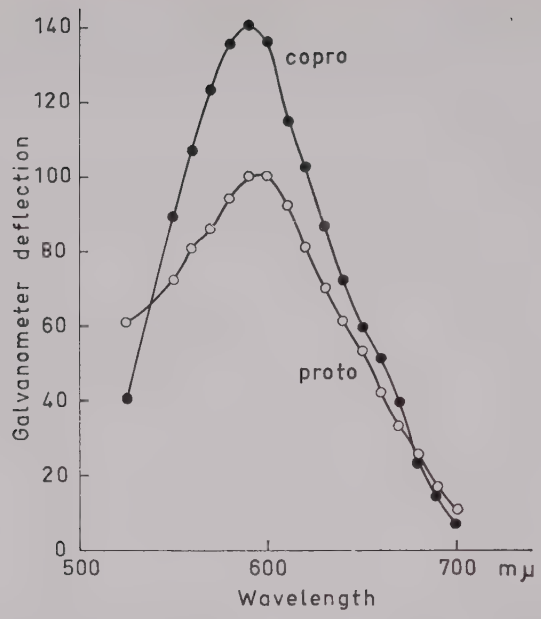

Fig. 2. Emission spectra of coproporphyrin (—●—●—●—) and protoporphyrin (—○—○—○—). 0.2 μg porphyrin/ml. Solvent: 0.2 *N* HCl.

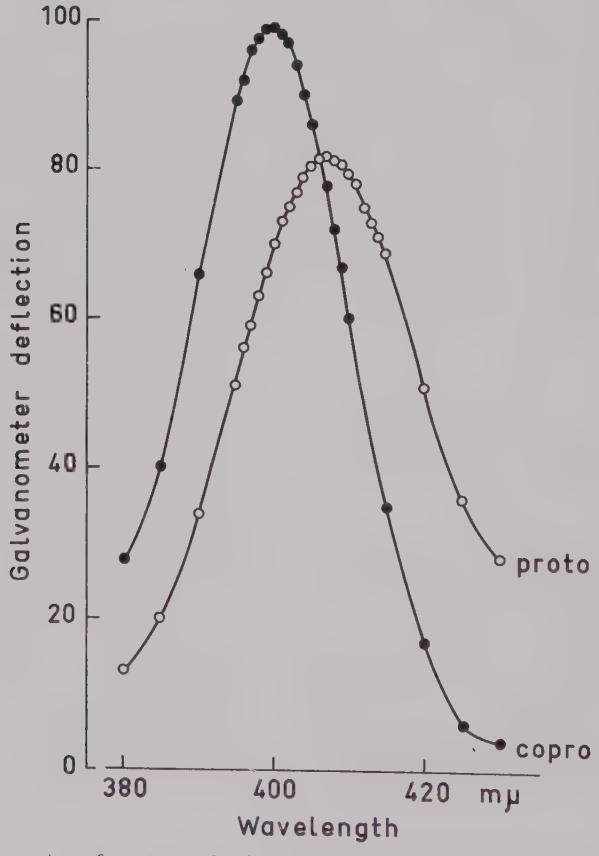

Fig. 3. Activation spectra of protoporphyrin (—○—○—○—) and coproporphyrin (—●—●—●—). 0.160 μg/ml. Solvent: 0.2 *N* HCl.

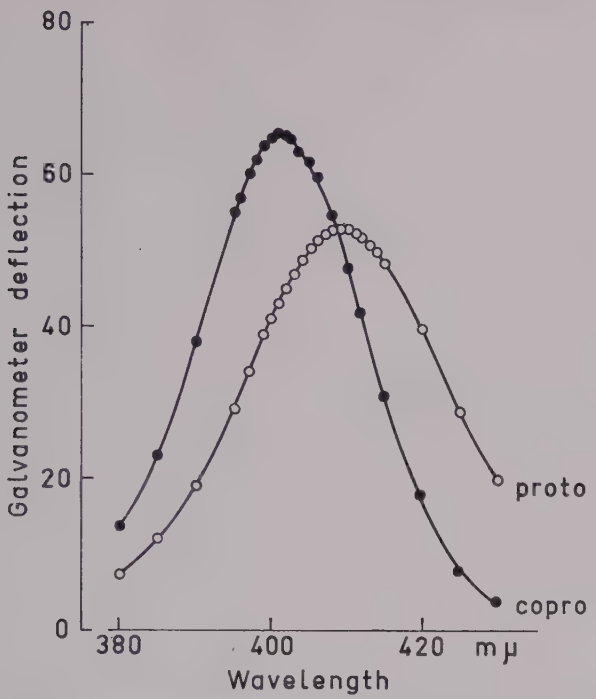

Fig. 4. Activation spectra of protoporphyrin (—○—○—○—) and coproporphyrin (—●—●—●—). 0.160 μg/ml. Solvent: 2 *N* HCl.

404.7 mμ line from a mercury lamp was isolated by aid of the monochromator. The fluorescent light was filtered through Jena filter OG 1, 2 mm, which transmits light of wavelengths above 510 mμ. The intensity of the fluorescence in the samples was compared to that of a coproporphyrin III standard.

Results

Porphyrin concentration. The fluorescence intensity, as judged from the galvanometric deflection, was found to be directly proportional to the concentration of coproporphyrin (Fig. 5). However, when higher concentrations than 0.1 μg coproporphyrin/ml was employed, the fluorescence was quenched.

Hydrochloric acid and sodium chloride concentrations. A standard concentration of coproporphyrin, 0.027 μg/ml was used. Hydrochloric acid and saline were added in varying concentrations. In Fig. 6, the suppression effect of increasing concentration of the acid is illustrated. In Fig. 7, the slightly less pronounced effect of NaCl is shown.

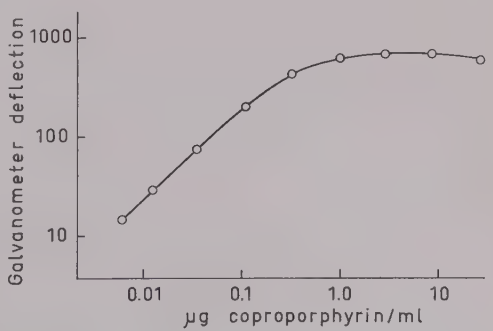

Fig. 5. The relation between coproporphyrin concentration and fluorescence intensity. Solvent: 0.2 *N* HCl.

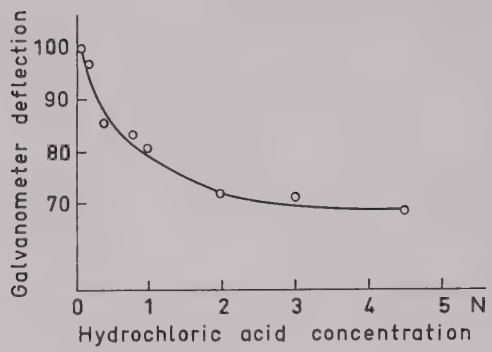

Fig. 6. The relation between coproporphyrin fluorescence and the concentration of hydrochloric acid in the solvent. 0.027 μg coproporphyrin/ml.

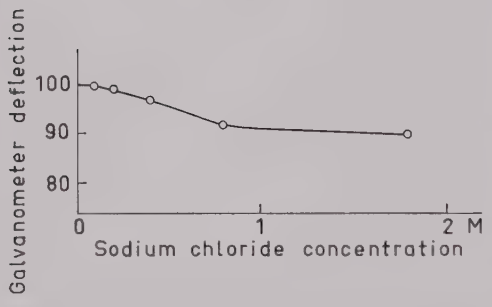

Fig. 7. The relation between coproporphyrin fluorescence and the concentration of sodium chloride in the solvent. 0.027 μg coproporphyrin/ml, dissolved in 0.2 *N* HCl.

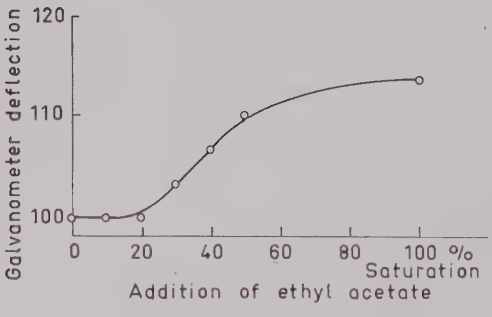

Fig. 8. The relation between coproporphyrin fluorescence and the degree of saturating the solvent with ethyl acetate. Room temperature. 0.027 μg coproporphyrin/ml, dissolved in 0.2 *N* HCl.

Ethyl acetate and acetic acid concentrations. The influence of different concentrations of ethyl acetate was studied using a 0.2 *N* HCl solution of 0.027 μg coproporphyrin/ml (Fig. 8). When the solution was saturated with ethyl acetate at room temperature, the fluorescence increased about 12 %. This was found to be true for other coproporphyrin concentrations as well. Addition of glacial acetic acid up to 20 % (*v*/*v*) was also studied and an increased fluorescence was noted (Fig. 9).

Fluorescence at varying pH. The influence of pH on fluorescence is difficult to evaluate owing to the double influence of buffers; pH as well as the individual constituents of the buffer may quench or enhance the intensity. In the present experiments, the desired pH was obtained by partial neutralization of HCl by sodium acetate. In this manner, conditions similar to those in the final 0.24 *N* HCl extracts were achieved. The fluorescence was found to be essentially unchanged between pH 0.85–1.2. The fluorescence was depressed markedly in solutions with pH higher than 1.7. In the final 0.24 *N* HCl extracts from a complete analysis, pH was found to be approximately 1 (Fig. 10).

Discussion

Emission and activation spectra. The present study seems to be the first report on the emission and activation spectra of proto- and coproporphyrin measured with a photoelectrical spectrofluorometer. When comparing with the earlier investigation on porphyrin emission spectra by Bois (1927), it is interesting to note that, in the present investigation, only one emission band could be found: the

maxima for proto- and coproporphyrin being 595 and 590 mμ, respectively. In the study of Bois, at least two bands were found (cf. Table 5). The explanation of this discrepancy is not clear. Obviously the emission spectra of proto- and coproporphyrin as obtained by the author's technique do not differ enough to permit a simultaneous estimation of the two porphyrins.

Earlier investigations of the activation spectra of copro- and protoporphyrin are limited to reports of the most favorable wavelength-regions for the activation of fluorescence. Thus Borst and Königsdörffer (1929) found the region 280–470 mμ and Bois (1927) 340–470 mμ to be most favorable. The present finding of sharp peaks in the activation spectra at the same wavelengths as the peaks of the different Soret bands are what could be expected from a theoretical point of view (Table 6). Accordingly, maximal fluorescence of pure proto- and coproporphyrin in 0.2 *N* HCl was elicited by light of the wavelengths 407 and 399.5 mμ.

Guided by these experiments, the routine fluorometric estimation was carried out in the following manner. The fluorescence was activated by the 404 mμ Hg line, isolated by the monochromator. The emitted light was separated from scattered light by a filter, transmitting less than 0.05 % at the wavelength of the activating light; 0.2 % at 509 mμ, 79 % at 546 mμ and 98 % at 578 and 644 mμ (Jena OG 1).

The concentration of the porphyrin. Fikentscher (1932) and Schwartz, Zieve and Watson (1951) have studied the relationship between fluorescence intensity and coproporphyrin concentration. In both

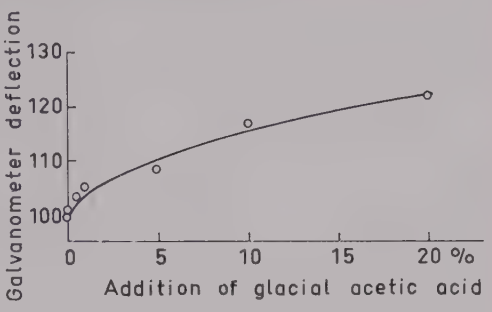

Fig. 9. The relation between coproporphyrin fluorescence and the concentration of acetic acid in the solvent. 0.027 μg coproporphyrin/ml, dissolved in 0.2 *N* HCl.

studies, a linear relation was found up to 0.5 and 0.05 μg/ml, respectively. Higher concentrations were followed by a deviation from linearity and finally by quenching of the fluorescence. The present finding of a linear relationship up to 0.1 μg/ml is confirmatory. The differences are probably due to differences in the fluorometers.

The influence of the solvent. Fluorescence intensity of coproporphyrin dissolved in hydrochloric acid, acetic acid and saline of various concentrations and the effect of saturating the solvent with

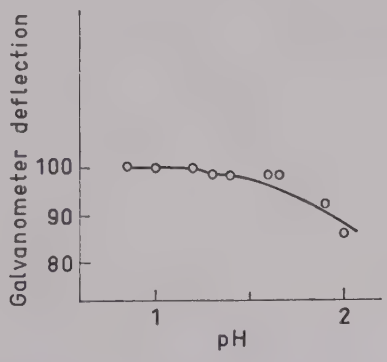

Fig. 10. The relation between coproporphyrin fluorescence and pH. 0.2 *N* HCl solutions were partially neutralized by 0.2 *M* sodium acetate to the desired pH. 0.013 μg coproporphyrin/ml.

TABLE 5. *Fluorescence emission bands of some porphyrins dissolved in 2 N HCl.* (Bois, 1927)

Band	I	II	III
Protoporphyrin	654		603
Coproporphyrin	652	618	595
Hematoporphyrin	653	619	597

Band I was stronger than bands II and III.

ethyl acetate has been only incompletely studied earlier. Schwartz, Zieve and Watson (1951) noted the suppression of coproporphyrin fluorescence in 1.5 *N* HCl compared to that in 0.3 *N* HCl; the ratio of fluorescence in 0.3 *N*/1.5 *N* HCl ranged between 1.10–1.20. Recently, Chu and Chu (1959) have published a study of porphyrin in which the coproporphyrin's fluorescence in varying HCl-concentrations was studied. According to the figures in their paper, the suppression of fluorescence was similar to the present findings.

The enhancement of the fluorescence when coproporphyrin solutions are saturated with ethyl acetate is important for the analysis of free erythrocyte coproporphyrin since ethyl acetate is always present in the final HCl-extracts. The present finding of a 12 % enhancement of the fluorescence of coproporphyrin dissolved in 0.2 *N* HCl is slightly higher than the 8 % enhancement found by Schwartz, Zieve and Watson (1951). However, the latter authors studied coproporphyrin dissolved in 1.5 *N* HCl. This may explain the difference.

The influence of various ions on the intensity of the porphyrin fluorescence is seldom mentioned in the literature. Fischer and Orth (1937) state that phosphate and citrate ions increase the fluorescence. Thus fluorescence-pH curves obtained by aid of Mac-Ilvaine's buffer published by Fink and Hoerburger (1935) and by Chu and Chu (1959) are not readily interpreted.

In the present experiments, mixtures of HCl and sodium acetate were used. The fluorescence was constant between pH 0.85–1.2, showing that the estimation of coproporphyrin is made under favorable pH conditions (pH of the final extracts being about 1). These findings do not completely agree with the findings of Jope and O'Brien (1945) who found a maximum fluorescence at pH 1.5. However, they do not state the composition of their buffers, and the results are therefore not comparable.

TABLE 6. *Wavelengths for maximum excitation of fluorescence compared to the wavelengths for maximum light absorption.*

	Coproporphyrin		Protoporphyrin	
	0.2 *N* HCl	2 *N* HCl	0.2 *N* HCl	2 *N* HCl
Maximum excitation	399–400	401	407	409
Maximum absorption	400	401–402	405	408

CHAPTER III

Losses of porphyrin during the analysis

During the preliminary experimental work it was found that losses of porphyrin occurred during the purification procedure. This was mainly due to the destruction of protoporphyrin by light and the formation of metal complexes of coproporphyrin. In addition to these findings, some other experiments are described concerning the stability of protoporphyrin in concentrated acid and the possible presence of reduced porphyrins in the erythrocyte samples.

1. Light sensitivity of protoporphyrin

The light sensitivity of protoporphyrin has long been known, and was noted by Lageder (1936) and by Grinstein and Watson (1943). The degradation products of protoporphyrin are illdefined and do not exhibit the same strong fluorescence and light absorption as the intact porphyrin. Thus porphyrin appears to be lost during the analysis.

To determine if the light sensitivity of protoporphyrin was important for the present work a series of experiments was performed (Tables 7 and 8). Equal amounts of protoporphyrin were dissolved in 1 *N* HCl and in ethyl acetate and left in diffuse daylight behind double layers of ordinary window-glass. After three hours, almost all protoporphyrin dissolved in the ethyl acetate and 90 % of protoporphyrin dissolved in HCl had disappeared when compared with controls kept in the dark. A corresponding experiment was performed, testing the stability of protoporphyrin kept in the dark and of protoporphyrin exposed to the subdued light from electric bulbs as used in the routine work. In this case no deterioration of the porphyrin could be detected (Table 7).

The importance of avoiding daylight was also studied by performing the complete analysis on samples of the same blood, at one occasion in strong daylight and at another occasion in subdued light from electric bulbs. When the analyses were performed in daylight, considerable loss of protoporphyrin occurred, as can be seen from Table 8. There was also a slight increase of the "coproporphyrin fraction".

TABLE 7. *The influence on light on the stability of protoporphyrin.*

	Solvent	
	1 *N* HCl μg	Ethyl acetate μg
After 3^h in daylight	0.16	0.04
Control	1.40	1.20
After 3^h in incandescent light	1.55	1.31
Control	1.53	1.32

TABLE 8. *Analyses of free erythrocyte proto- and coproporphyrin during different light conditions. Four analyses of each sample of erythrocytes.*

	Protoporphyrin μg/100 ml RBC	Coproporphyrin μg/100 ml RBC
Strong daylight	4, 6, 10, 13	1.8, 2.9, 1.7[a]
Incandescent light	21.5, 21.5, 22.1, 24.5	0.9, 1.0, 0.8, 1.1

[a] one analysis was lost.

This is explained, perhaps, by accelerated formation of hematoporphyrin-like pigments.

In order to minimize protoporphyrin loss due to light destruction, the analyses were performed in subdued light from electric bulbs. Daylight was avoided as far as possible. During intermissions in the analytical work, separating funnels and other vessels containing porphyrin solutions were covered with black cloth.

2. The possible formation of metal complex

Porphyrins are known to form complexes with metals. Oliver and Rawlinson (1951) studied a copper complex of coproporphyrin. Since this complex does not fluoresce, the complex formation was used for the quantitative estimation of small amounts of coproporphyrin. Zinc also forms complexes very readily with porphyrins. Brugsch (1959) states that the complexes are formed even at normal temperature and in dilute acid solutions. Thus serious errors in the determination of minute amounts of coproporphyrin can be introduced.

In the present investigation, it was found difficult to obtain quantitative recovery of coproporphyrin added to ethyl acetate and subsequently extracted by dilute hydrochloric acid. It was suspected that a formation of porphyrin-metal complexes had occurred although the presence of metals could not be proved. The addition of EDTA[1] to all batches of ethyl acetate was followed by satisfactory recoveries and no untoward effects could be observed. The EDTA was added in substance to the ethyl acetate, shaken and allowed to settle. The ethyl acetate was then decanted.

3. Protoporphyrin degradation in strong acid

Schumm (1927) established that protoporphyrin dissolved in strong acid is partially transformed into hematoporphyrin. According to Paul (1953), it is possible that this transformation occurs via an intermediate; i.e., monovinyl, monohydroxyethyl, tetramethyl, porphin dipropionic acid. The hematoporphyrin is extracted in the coproporphyrin fraction and may introduce severe errors in the estimation of free erythrocyte coproporphyrin. There does not seem to exist any practical method for the separation of hemato- and coproporphyrin in these small concentrations (Brugsch 1959). During the analysis of free erythrocyte proto- and copropor-

[1] "Titriplex III", the disodium salt of ethylenediaminotetraacetic acid.

phyrin, rather concentrated hydrochloric acid has to be employed; thus it was considered important to try to ascertain if protoporphyrin was transformed to hematoporphyrin or hematoporphyrin-like pigments during the analysis. This was thought to be particularly important since studies of this kind had not been reported earlier.

Protoporphyrin dimethyl ester was hydrolyzed overnight in 7 *N* HCl, and subsequently diluted to 1 *N* HCl. In order to remove all traces of hematoporphyrin and other pigments with low HCl-numbers, the protoporphyrin was purified by repeated distributions between 0.24 *N* HCl and ethyl acetate according to the principles of Paul (1953). The protoporphyrin goes into the ethyl acetate phase, and the hematoporphyrin-like pigments accumulate in the hydrochloric acid. This purification was done immediately before the protoporphyrin was added to the first stage of the complete analysis. Ten experiments of this kind were carried out. The added amounts corresponded to 10–250 μg % free erythrocyte protoporphyrin. After performing all the usual steps of the analysis, protoporphyrin and "coproporphyrin" were determined in the routine manner. The "coproporphyrin" fractions showed a fluorescence which would indicate -0.006 to $+0.028$ μg coproporphyrin (owing to the error of the fluorometric analyses, some results were "negative"). Cf. Table 9.

Discussion. It has not been possible to study in closer detail the formation of hematoporphyrin from protoporphyrin. Probably a number of other degradation products are formed in addition to hematoporphyrin. However, when relatively small amounts of protoporphyrin (less than 3 μg, corresponding to about 100 μg % free erythrocyte protoporphyrin) are present, only insignificant amounts of coproporphyrin-simulating fluorescence appears in the 0.24 *N* HCl extracts. The coproporphyrin values become too high with blood samples containing more than about 100 μg % protoporphyrin. The increase has the magnitude of 0.3–0.4 μg % coproporphyrin per 100 μg % protoporphyrin.

TABLE 9. *Protoporphyrin degradation to hematoporphyrin-like pigments.*

Protoporphyrin found, μg	Fluorescence in 0.24 *N* HCl extracts corresponding to coproporphyrin, μg
0.35	+0.003
0.41	−0.0007
0.59	−0.006
0.77	−0.0015
0.98	+0.012
1.21	+0.0005
3.14	+0.0025
3.18	+0.012
6.27	+0.028
6.28	+0.026

4. Reduced porphyrins

Fischer, Bartholomäus and Röse (1913) established that reduction of a porphyrin with sodium amalgam yields a colorless substance which easily could be reoxidized by air. Studying the porphyrin in urine, Watson, de Mello, Schwartz, Hawkinson and Bossenmaier (1951) found a coproporphyrin precursor similar to a reduced coproporphyrin. The precursor was easily extracted by ethyl acetate but from this solution only insignificant amounts could be extracted by hydrochloric acid. The precursor was completely oxidized by

TABLE 10. *Comparison of porphyrin yield from samples of erythrocytes analyzed with and without iodine wash.*

	Number of pairs	Mean results μg/100 ml RBC Without I_2-wash	With I_2-wash
Protoporphyrin	11	26.4	27.7
Coproporphyrin	13	1.3	1.1

dilute iodine solutions. Neve, Labbe and Aldrich (1956) and, later on, several other investigators have demonstrated that not the porphyrins themselves but the reduced analogues of uro- and coproporphyrin are intermediates in the synthesis of protoporphyrin and heme. The reduced analogues are often called "porphyrinogens".

During the study of the method for the estimation of free erythrocyte proto- and coproporphyrin, it was considered important to try to demonstrate the presence or absence of porphyrinogens in the erythrocytes—particularly coproporphyrinogen. In accordance to the findings of Watson *et al.* (1951) the ethyl acetate/glacial acetic acid extract of the erythrocytes was washed with a fresh aqueous solution of iodine (0.005 %) in order to oxidize any porphyrinogens present. Duplicate samples of blood from healthy individuals were studied and analyzed with the routine method adopted by the author as well as with this method completed with the iodine wash. Results are found in Table 10. No significant differences were found.

This result cannot be taken as evidence of the absence of porphyrinogens in the erythrocytes since the porphyrinogens are known to be easily autoxidized when exposed to air. During the course of the extraction procedure, such oxidation may easily have taken place. However, the results indicate that no porphyrinogens escape the analysis as was the case in the urine analysis of Watson *et al.* (1951).

CHAPTER IV

Extraction and purification of free erythrocyte copro- and protoporphyrin

1. Extraction of the erythrocytes

In the first step of the analysis, the erythrocytes are treated with a mixture of ethyl acetate and glacial acetic acid in order to extract the free porphyrins. The precipitate formed is thoroughly mixed with the extraction mixture. The extract is removed by filtration and the precipitate washed three times by small amounts of the extraction mixture.

In an attempt to ascertain the efficiency of the extraction of free erythrocyte porphyrins, the following experiment was performed. Samples of erythrocytes were extracted as described above and the content of free proto- and coproporphyrin determined. In order to find out if the precipitate still contained porphyrin, a second extraction was performed in the same manner. The free protoporphyrin in the second extraction was found to be 2–5 % of that of the first extraction. The free coproporphyrin in the second extraction appeared to be insignificant (Table 11).

The result of these experiments indicates that no important amount of proto- or coproporphyrin remains in the precipitate after one routine extraction. This is in accordance with the results of Ward and Mason (1950) who in a similar experiment found 2–3 % in the second extract. These authors did not study the coproporphyrin content of the erythrocytes.

2. Possible formation of free protoporphyrin from hemoglobin

According to Schumm (1924), protoporphyrin is split off from hemoglobin much more easily than from oxyhemoglobin. In a later paper (1939), the same author emphasized the importance of using oxygenated blood for the analysis of free porphyrin in plasma. The necessity of a similar precaution is not mentioned in the discussion of the analysis of free erythrocyte porphyrin (1939) although in the earlier paper (1924) it is reported that porphyrin can be obtained in great amounts from blood which had been stored in sterile vessels at room temperature until it acquired the colour of reduced hemoglobin.

A formation of protoporphyrin from hemoglobin during the analysis of free red-cell porphyrin might form a serious source of error. Van den Bergh and Hyman (1928) tried to disprove the possibility of protoporphyrin formation by extracting crystalline hemoglobin. No protoporphyrin was found in the extract. Later,

TABLE 11. *Repeated extraction of a number of erythrocyte samples.*

Erythrocyte sample number	Porphyrin in the first extract μg	Porphyrin in the second extract μg	Porphyrin in second extract in per cent of first extract
Protoporphyrin			
1	0.96	0.02	2.1
2	1.16	0.05	4.3
3	1.17	0.05	3.6
4	1.61	0.08	5.0
5	4.51	0.17	3.8
6	5.44	0.16	2.9
Coproporphyrin			
7	0.011	+0.0016	—
8	0.049	−0.002	—
9	0.052	0.000	—

several authors have discussed the possibility of protoporphyrin formation from hemoglobin during the analysis—most thoroughly perhaps Bénard, Gajdos and Canivet (1949). However, the risk was considered insignificant. According to Brugsch (1959), the risk of protoporphyrin formation from hemoglobin is most important when blood or parts of organs from corpses are extracted by mineral acids.

During the present work, determinations of free protoporphyrin were performed on four samples of blood, two of which had been repeatedly shaken in nitrogen atmosphere and two shaken in air. No difference was found between the two pairs when subsequently analyzed for the presence of free protoporphyrin. In the present investigation, as in those of most authors, no special precautions were taken to ensure maximum oxygenation of the blood sample.

3. The distribution of porphyrin between ethyl acetate and hydrochloric acid

The distribution of porphyrins between ether and hydrochloric acid at varying concentrations of the latter is often given by their "HCl-number". The "HCl-number" is defined as the concentration of hydrochloric acid in per cent which from an equal volume of ether solution of a porphyrin extracts two-thirds of the porphyrin. The "HCl-number" of protoporphyrin is 2, of coproporphyrin 0.09 and of hematoporphyrin 0.1. When porphyrins are distributed between ethyl acetate and hydrochloric acid, the corresponding HCl-numbers are higher. Under the conditions employed in the present study, they were found to be 8 for protoporphyrin and 0.4 for coproporphyrin. More concentrated acid is thus necessary for extractions from ethyl acetate than from ether. Ethyl acetate was preferred for various practical reasons. Schwartz and Wikoff (1952), in their method for the determination of free erythrocyte copro- and protoporphyrin, also use ethyl acetate instead of ether.

Considering the HCl-numbers of proto- and coproporphyrin, the separation would be expected to be easy. However, the fact that protoporphyrin occurs in much higher concentrations than those of coproporphyrin (about 20–50 μg protoporphy-

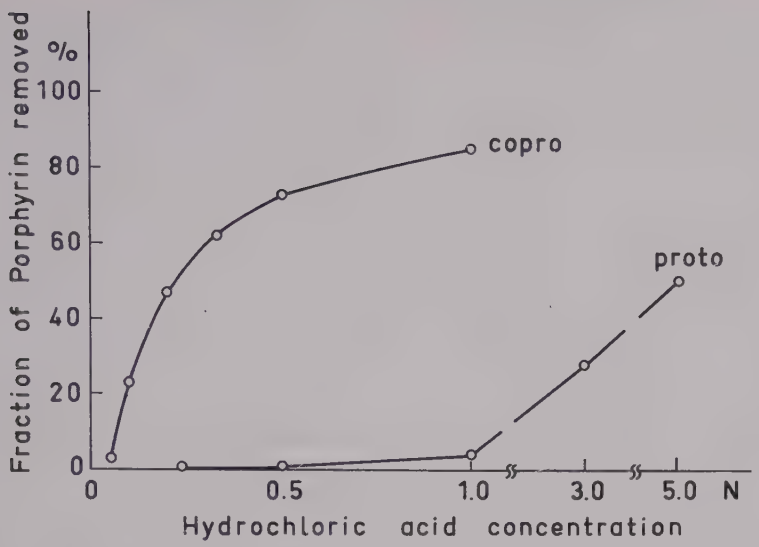

Fig. 11. The fraction of copro- or protoporphyrin removed from 150 ml water-saturated ethyl acetate solution by one extraction with 4 ml HCl of different concentrations.

rin and about 0.5–2 μg coproporphyrin per 100 ml erythrocytes) makes the selection of a suitable HCl-concentration very important.

The distribution of proto- and coproporphyrin between water-saturated ethyl acetate and hydrochloric acid was further studied. The volumes of the two solvents were chosen to be similar to those used in the final extraction of porphyrins from ethyl acetate solution by Schwartz and Wikoff (1952). The exact volumes of solvents are not stated in their paper which makes a strict comparison impossible. The volumes were equal to those used for the routine determination of erythrocyte proto- and coproporphyrin (150 ml ethyl acetate are extracted by 4 ml hydrochloric acid, cf. p. 12). Known amounts of copro- or protoporphyrin were added to the water-saturated ethyl acetate. Extraction was performed once by hydrochloric acid. The extracts were made up to the same volume and adjusted to the same HCl concentration by adding water and HCl. The amounts extracted were calculated according to With (1955) after measuring the absorption in near ultraviolet. The experiments revealed (Fig. 11) that relatively high concentrations of HCl are necessary to obtain a satisfactory removal of the porphyrins by a limited number of extractions.

Discussion

Protoporphyrin. Earlier workers have used a wide range of HCl concentrations for the extraction of protoporphyrin. Thus in the original description by van den Bergh (1928), 5 % HCl (about 1.7 *N*) is recommended, while Schumm (1939) uses the more efficient 25 % HCl (about 8 *N*) for extraction. Schwartz and Wikoff (1952) use 3 *N* HCl. In the present investigation, the latter concentration was found rather inefficient; at least 5 *N* HCl had to be ap-

plied in order to avoid too many extractions. Higher concentrations of the acid were not used in order to minimize the formation of hematoporphyrin-like pigments.

Coproporphyrin. For the extraction of coproporphyrin in the presence of protoporphyrin, it is important not to use too high acid concentrations in order to avoid unnecessary co-extraction of protoporphyrin. Schwartz and Wikoff (1952) used 0.1 *N* HCl, but this concentration obviously extracts too small fractions of the coproporphyrin. In spite of the high dilution of the acid, traces of protoporphyrin were extracted and had to be removed by washings with chloroform. About 50 % of the coproporphyrin is removed by one extraction with 0.24 *N* HCl; simultaneously 0.25 % or slightly less of the protoporphyrin is co-extracted. Since the ratio between proto- and coproporphyrin generally is 20–40/1, sometimes 100/1, $\frac{1}{4}$ to $\frac{1}{2}$ of the fluorescence of the 0.24 *N* HCl extracts can be expected to come from protoporphyrin. It was not considered advisable to increase this fraction by using stronger concentrations of the acid.

According to the theory of distribution, only one extraction under controlled conditions ought to be necessary; and the total amount of porphyrin can be calculated by aid of distribution coefficients. However, calculations based on distribution coefficients cannot be strictly applied here. The water in the water-saturated ethyl acetate dilutes the acid. During routine conditions 5 *N* HCl was diluted to about 3 *N* and 0.24 *N* HCl to about 0.20 *N*. The separation of the two phases takes a long time to be perfect. A small but significant amount of the acid extract is left in the stopcock and often also in droplets on the walls of the funnel. Even if four consecutive extractions are made, it is always necessary to carry out the analysis with a carefully standardized technique in order to obtain satisfactory reproducibility.

Removal of protoporphyrin from coproporphyrin solutions by chloroform extractions. Schwartz and Wikoff (1952) recommend the washing of the coproporphyrin extracts by chloroform "to remove traces of chloroform-soluble porphyrins". Trying this method, the present author found it troublesome, disturbing the subsequent fluorometric estimation of coproporphyrin and also rather inefficient even if large amounts of chloroform were used. The ratio between the volumes used by Schwartz and Wikoff is not given exactly, but probably about 0.25 volume of chloroform to one volume of the 0.1 *N* HCl extract has been used. During the present study, the effect of washing with 0.5 volume of chloroform was tried. Only 45 % of the protoporphyrin was removed by each washing and more than three washings would be necessary to remove at least 85 % of the protoporphyrin.

4. The background fluorescence and the correction for traces of protoporphyrin in the final 0.24 *N* HCl extracts

When small intensities of fluorescence are studied, attention must be paid to the possible presence of a background reading on the galvanometer in addition to the usual "dark current" reading. This background is composed of scattered light and of fluorescence that does not come from the investigated fluorescent substance. Considerable reduction of the background

may be achieved by using the purest chemicals, perfectly clear test solutions, and by extreme cleanliness when handling the cells and other parts of the optical system. Also, to diminish the effects of the scattered light, it is very important to choose suitable wavelength-ranges of the exciting and emitted light.

Paying attention to the factors mentioned above, the background galvanometer readings were studied in some solvents. (The galvanometer was adjusted to zero when the exciting light was shut off.) As can be seen from Table 12, the background readings of distilled water and dilute hydrochloric acid are negligible, while that of ethyl acetate is more noticeable. Re-distillation *in vacuo* diminished the background of ethyl acetate to 50 %. Dilute hydrochloric acid saturated with ethyl acetate showed a moderate background that was not noticeably diminished by re-distilling the ethyl acetate.

In the final extracts of the analysis of free erythrocyte coproporphyrin, the intensity of the background fluorescence was found difficult to estimate, since it appeared to vary from one analysis to another. When attempting to measure quantitatively the fluorescence of coproporphyrin, another difficulty was encountered, viz. the presence of protoporphyrin in the 0.24 *N* HCl extracts. Attempts to remove the protoporphyrin by washing with chloroform according to Schwartz and Wikoff (1952) had not been successful and some other method was sought for. The combined problem of correcting for the background and the protoporphyrin fluorescence was studied from various aspects, and finally the following procedure was adopted. Six consecutive extractions are performed with 0.24 *N* HCl. According to earlier experiments each extraction removes about 50 % of the coproporphyrin and less than 0.25 % of the protoporphyrin present in the ethyl acetate solution. The sixth extract is practically free from coproporphyrin and the fluorescence of that extract is taken as a measure of the background and of the protoporphyrin fluorescence. Assuming that the protoporphyrin and the factors giving rise to the background fluorescence are constant in the six consecutive extracts, the coproporphyrin fluorescence can be calculated.

TABLE 12. *"Fluorescence's background" of some solvents.*

Solvent	Equivalent to coproporphyrin $\mu g \cdot 10^{-2}$/ml
Distilled water	1.5
0.2 *N* HCl	2
Ethyl acetate	10
Ethyl acetate, distilled in vacuo	5
0.2 *N* HCl, saturated with ethyl acetate	3.5
0.2 *N* HCl saturated with distilled ethyl acetate	3.5

The sixth extract is not, of course, completely free of coproporphyrin; however, the amount can be calculated as follows. Let k be the fraction of coproporphyrin that remains in the ethyl acetate phase after one extraction with hydrochloric acid. After two extractions, k^2 remains and after six extractions k^6. Correspondingly, in the sixth hydrochloric acid extract a fraction $k^5(1-k)$ appears. During the conditions used in the analysis, k is approximately equal to 0.5. If k equals 0.50, the first five hydrochloric acid extracts contain a total of 97 % and the sixth extract 1.56 % of the coproporphyrin. If the last extract is considered copropor-

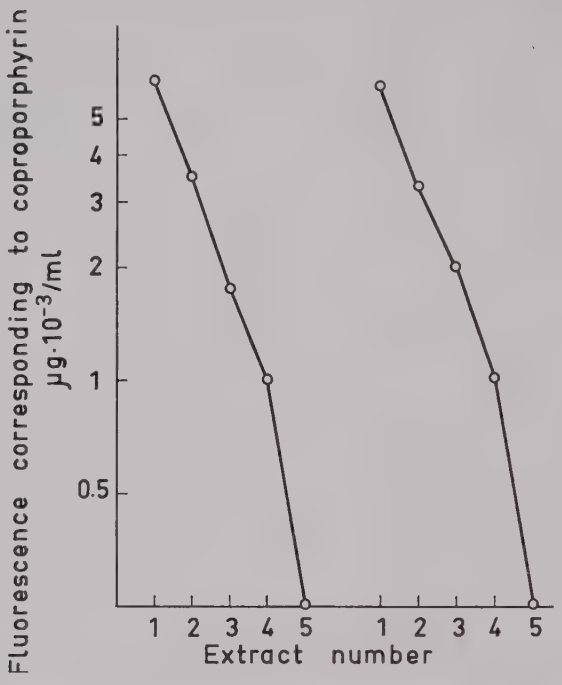

Fig. 12. Fluorescence intensities in the six consecutive 0.24 *N* HCl extracts. The fluorescence of the sixth extract was subtracted from the fluorescence of each of the preceding extracts. Two different samples of erythrocytes.

phyrin-free an error of 1.56 ×7 or 10.9 % is introduced and the calculated amount of coproporphyrin is about 89 % of the true amount.

The error introduced in the determination of coproporphyrin by the assumption that protoporphyrin fluorescence is constant in the consecutive extracts is very small. If 0.250 % of the protoporphyrin goes into the first hydrochloric acid extract, the sixth extract will contain 0.247 %. Even if the free erythrocyte protoporphyrin is high, e.g. 300 µg %, the result of the free erythrocyte coproporphyrin analysis is only increased about 0.03 µg %.

Of course, these calculations are only valid if the distribution coefficients for proto- and coproporphyrin, the fluorescence from other substances and the light scattering are constant. In practice, the experimental results supported the theoretical consideration. The calculated intensity of the coproporphyrin fluorescence plotted against the extraction number yielded almost straight lines on a semilogarithmic diagram (Fig. 12).

5. The enhancement of fluorescence in the six consecutive 0.24 *N* HCl extracts

The method adopted for the determination of coproporphyrin is justified only if, in addition to the enhancement caused by ethyl acetate, no other factors influence the intensity of the fluorescence of the six extracts. In order to study this question, the following experiment was carried out. Six samples of normal erythrocytes were examined by the routine ana-

TABLE 13. *The enhancement of fluorescence by the 0.24 N HCl extracts.*

Ordinal number of extract	Expected increase of fluorescence Arbitrary units	Observed increase of fluorescence Arbitrary units
1st	100	102
2nd	100	115
3rd	100	112
4th	100	115
5th	100	108
6th	100	108

lysis of free erythrocyte proto- and coproporphyrin. The first 0.24 *N* HCl extracts from the six analyses were pooled, the second extracts pooled, and so on. The fluorescence of the contents of each of the pools was measured and a small volume of a standard coproporphyrin solution added. The fluorescence increase was then measured and compared with the expected increase. The background fluorescence was small compared to the fluorescence brought about by the addition.

In all extracts, the coproporphyrin fluorescence increased more than expected. In the first extract, this was less pronounced than in the subsequent ones (Table 13). If no other factor than the saturation with ethyl acetate was responsible, the observed fluorescence should be 12 % higher than the expected (cf. p. 20). This would also be the case in the last five extracts. An increased NaCl concentration in the first extract is one possible cause of the less pronounced enhancement of the fluorescence in the first extract. Since the fluorometric estimation was always corrected for the 12 % enhancement of the coproporphyrin fluorescence, the less pronounced enhancement in the first extract causes a total underestimation of coproporphyrin by 4–5 %.

6. Blank values of the complete determination of free erythrocyte proto- and coproporphyrin

During the methodological studies, methods were elaborated for the reduction of the influence of impurities and other "blank" factors. In an attempt to ascertain if these factors were, in fact, satisfactorily reduced, ten "blank" determinations were performed without any addition of erythrocytes or plasma. As can be seen from Table 14, the "blank" light absorption of the 5 *N* HCl extracts was negligible, corresponding on the average to 0.02 μg protoporphyrin. Even when using the method described earlier (p. 31) for the correction of the fluorometrical determination of coproporphyrin, fluorescence intensities were found in the 0.24 *N* HCl extracts corresponding on the average to 0.0056 μg coproporphyrin.

Earlier, the presence of "blanks" in the determination of free erythrocyte proto-

TABLE 14. *Results of ten blank determinations of free erythrocyte proto- and coproporphyrin.*

	Light absorption corresponding to protoporphyrin μg	Fluorescence corresponding to coproporphyrin μg
Range	-0.025 to $+0.055$	$+0.0011$ to $+0.0094$
Mean $\pm e\,(M)$	0.02 ± 0.008	0.0056 ± 0.0009

porphyrin has been described by Sturgeon (1954). With his method, the mean light absorption equalled 4 μg protoporphyrin. With the present method, a negligible protoporphyrin "blank" was found. This favorable result is thought to be due to the use of the Rimington–Sveinssen correction (p. 14). No correction for the small protoporphyrin "blank" has been performed.

However, in the determination of coproporphyrin, a "blank" was found that could not be disregarded. The cause of this "blank" is not clear. In most cases, the "blank" has been found to be due to an "extra fluorescence" appearing in the first extract. It is probable that the "fluorescence" of this extract contains more scattered light than the rest. Thus the blank correction made by the subtraction of the "fluorescence" in the sixth extract becomes insufficient. It should be noted, that in the experiment just described (Table 13), the "extra fluorescence" was very small compared to the fluorescence of the added coproporphyrin. In Fig. 12, p. 32, the "extra fluorescence" is not observed since its effect is opposed by the depressing of the fluorescence in the first extract.—In all determinations, the sum of the coproporphyrin fluorescence in the first five extracts was corrected by subtracting 0.0056 μg.

CHAPTER V

Evaluation of the method

1. Recovery experiments

Recovery experiments were made as follows: Hydrochloric acid solutions of copro- or protoporphyrin were partially neutralized with sodium acetate. The porphyrins were transferred quantitatively into ethyl acetate. Aliquots of the ethyl acetate solution were added to the mixture of ethyl acetate and glacial acetic acid used for the extraction of erythrocytes. In some cases, the recovery from pure solutions without erythrocytes was also studied. The volumes of the added solutions were small compared to the volume of the ethyl acetate/acetic acid mixture. The analysis was completed and the recovery of the added porphyrin calculated. In separate samples of the erythrocyte, the preformed content of free porphyrins was determined.

Results

Table 15 shows the results of coproporphyrin and protoporphyrin additions. The coproporphyrin recoveries ranged between 51 and 90 %, averaging 74 %. Protoporphyrin recoveries varied between 68 and 91 %, averaging 78 %. Slightly higher recoveries of coproporphyrin were obtained from the additions to pure solutions than from additions to erythrocyte extracts. In contrast, the recoveries of protoporphyrin additions to erythrocytes extracts were better than the recoveries from pure solutions.

Discussion

Recovery experiments of free erythrocyte copro- and protoporphyrin, independently, have apparently not been reported earlier. Schwartz and Wikoff (1952) who have described the only method to date, do not list any recovery experiments. However, these experiments have been carried out in the analysis of free erythrocyte porphyrin without differentiation between copro- and protoporphyrin. Since most of the erythrocyte porphyrin consists of protoporphyrin, a comparison may be made with the present recovery experiments with protoporphyrin.

The method of Grinstein and Wintrobe (1948) has been investigated by them and also by Ventura (1954 a). The highest recovery of added protoporphyrin was reported by Ventura, averaging 92 %. Grinstein and Wintrobe recovered 84–96 %, averaging 90 %. The latter authors also studied the recovery of protoporphyrin added to pure solutions, and similar results were obtained.

In the present author's experiments, the recoveries are somewhat lower. However,

TABLE 15. *The recovery of added copro- and protoporphyrin.*

Exp. No.	Preformed porphyrin μg	Added porphyrin μg	Porphyrin found μg	Recovery of added porhyrin μg	Per cent added porphyrin recovered
Coproporphyrin					
1	—	0.054	0.045	0.045	83
2	—	0.108	0.086	0.086	79
3	—	0.162	0.135	0.135	81
4	0.025	0.054	0.065	0.040	74
5	0.025	0.108	0.082	0.057	53
6	0.025	0.162	0.112	0.087	54
7	0.038	0.200	0.208	0.170	85
8	0.038	0.400	0.370	0.332	83
9	0.038	0.600	0.580	0.542	90
10	0.036	0.200	0.139	0.103	51
11	0.036	0.400	0.343	0.307	77
12	0.036	0.600	0.532	0.496	83
Protoporphyrin					
13	—	0.40	0.28	0.28	70
14	—	0.80	0.54	0.54	68
15	—	1.20	0.92	0.92	74
16	1.00	0.65	1.59	0.59	91
17	1.00	1.29	2.14	1.14	87
18	1.00	1.94	2.69	1.69	86
19	0.85	0.65	1.31	0.46	71
20	0.85	1.29	1.77	0.92	71
21	0.85	1.94	2.50	1.65	85

the methods are not fully comparable. Grinstein and Wintrobe used ethyl ether instead of ethyl acetate in the purification of the porphyrin. In addition, they estimated the protoporphyrin content by measuring the maximum light absorption in the Soret band, while in the present study the correction suggested by Rimington and Sveinssen is used.

The expected losses of protoporphyrin during the analysis according to the present method can be estimated to 15–20 %, viz. 6–7 % at each of the two extractions of the organic solvent by acid and a few per cent or less at the other extractions and washings. Thus the observed recovery, 78 %, is only slightly lower than the expected. The difference is probably dependent on inevitable light destruction. The fraction of protoporphyrin recovered varies between 68 and 91 %. This variation is consistent with the standard error of a single determination as calculated from 20 duplicate determinations of free erythrocyte protoporphyrin (see later). According to these analyses, the variance was 4.4. The variance of the present recovery experiments is 5.0.

The method described by Grinstein and Watson (1943) for the analysis of free erythrocyte porphyrin was modified by Schwartz and Wikoff (1952) to permit a simultaneous determination of copro- and protoporphyrin. The modification was based on a method intended for the analysis of *urinary* coproporphyrin described by Schwartz, Zieve and Watson (1951). This method involves extraction of the

coproporphyrin with buffered ethyl acetate washing with sodium acetate and finally extraction with 1.5 *N* HCl. No further purification was carried out. The amount of coproporphyrin was determined by a fluorometer. The recovery of added coproporphyrin was very satisfactory, averaging 100 %.

When this method was applied to the erythrocyte coproporphyrin, Schwartz and Wikoff (1952) introduced a purification step according to van den Bergh and Hyman and extracted the coproporphyrin from the ethyl acetate by 0.1 *N* HCl. These modifications tend to decrease the recovery of added coproporphyrin; and thus comparison of the recoveries should not be made between the present method and the method for the analysis of urinary coproporphyrin.

In the present method, the theoretical loss of coproporphyrin during the final extraction with 0.24 *N* HCl and the subsequent estimation has been calculated earlier to 15 %. The losses during the other steps in the analysis are small—less than a few per cent in each step. The theoretical recovery of the complete analysis is thus about 80 %. The recovery found when additions were made to pure solutions averaged 81 %. This would indicate that other losses were insignificant. However, when additions were made to extracts of erythrocytes, the recovery averaged 72 %. No certain explanation of this loss can be given. It may be partly due to formation of metal complexes.

In the determinations of free erythrocyte porphyrin, the results were corrected according to the findings in the recovery experiments. For practical reasons, the average *protoporphyrin* recovery was rounded off to 80 %. All results were therefore multiplied by a factor 1.25. In a similar manner, the results of determinations of free erythrocyte *coproporphyrin* were multiplied by 1.33.

In order to clarify the description of the calculations, the following example is described. Volume of the blood sample = 8 ml. Hematocrit = 35 %. Hence erythrocyte volume = 2.8 ml. Volume of combined 5 *N* HCl-extracts = 25 ml. Spectrophotometrical readings (5 cm cells): Extinction at the Soret band peak = 0.076, at 380 mμ = 0.022, at 430 mμ = 0.020. Hence $2E_{max} - E_{380} - E_{430}$ = 0.110. With's coefficient for protoporphyrin (1 cm cells) = 1.225. Hence protoporphyrin concentration = 0.027 μg/ml. Total volume of extracts 25 ml, and thus total protoporphyrin amount in extracts = 0.67 μg. Calculated protoporphyrin amount in sample = 0.84 μg (80 % efficiency of the analysis). Thus free erythrocyte protoporphyrin = 30 μg %. Fluorescence of the six 0.24 *N* HCl extracts = 16, 12.5, 11.5, 10.5, 10, 9.5 scale divisions, each corresponding to 0.5 $\mu g \cdot 10^{-3}$ coproporphyrin/ml. Fluorescence considered to be due to coproporphyrin = 6.5, 3, 2, 1.0, 0.5, 0 scale divisions. Volume of the first five extracts = 4.5, 5.0, 5.2, 4.8, 4.8 ml. Hence coproporphyrin content of the extracts = 14.6, 7.5, 5.1, 2.4, 1.2 $\mu g \cdot 10^{-3}$, in all 30.8 $\mu g \cdot 10^{-3}$. After subtraction of the blank of the complete determination (5.6 $\mu g \cdot 10^{-3}$), the total coproporphyrin amount in the extracts is 25.2 $\mu g \cdot 10^{-3}$. Calculated coproporphyrin in sample 33.6 $\mu g \cdot 10^{-3}$ (75 % efficiency of the analysis). Thus free erythrocyte coproporphyrin 1.2 μg %.

2. Error of the analysis

Duplicate analyses were performed on blood samples from 20 healthy subjects and on plasma samples from 10 subjects. Three to four ml of erythrocytes or 5 ml

plasma were used for each determination.[1] The standard error of a single determination of erythrocyte protoporphyrin was found to be ± 2.6 μg %; of erythrocyte coproporphyrin, ± 0.23 μg %; of plasma porphyrin, ± 0.25 μg %.

Discussion

The final error of analysis is probably composed of several factors such as: varying losses during the extraction and purification of the porphyrins; a certain destruction of the porphyrins by light, by strong acid or by metal complex formation; variations in the blank values; errors in the physical determination of the porphyrins in the final extracts. The two factors mentioned last have been possible to estimate, approximately. They were found to represent about one third of the total error of protoporphyrin analysis and about half the total error of coproporphyrin analysis.

Earlier reports about the errors in the analysis of free erythrocyte copro- and protoporphyrin have not been published. However, in three communications, the error of erythrocyte porphyrin analyses without differentiation of proto- and coproporphyrin has been studied. In the reports of Sturgeon (1954) and Koskelo (1959), the results are in agreement with those of the author. Sturgeon found a percentual standard error of ± 6 % and Koskelo an absolute standard error of ± 3 μg %. Ward and Mason (1950) report a very small standard error of ± 2.5 %. It is difficult to explain why the latter authors have obtained a so much better result since the methods of extraction and purification of the porphyrins were essentially identical. Sturgeon and Ward and Mason used the technique proposed by Grinstein and Watson (1943) and Koskelo the micro-modification by Grinstein and Wintrobe (1948). But the methods of estimating the porphyrin content in the final extracts were different. Ward and Mason measured the fluorescence intensity; Sturgeon studied the maximal light absorption in the Soret peak; and Koskelo also made readings of the light absorption at 380 and 430 mμ according to the principles of Rimington and Sveinssen (1950). According to the author's experience with free erythrocyte protoporphyrin, the fluorometric analysis is not more reliable than the spectrophotometric one, and therefore Ward and Mason's better results are difficult to explain.

3. The identification of the free erythrocyte porphyrins

During recent years, a number of intermediates in protoporphyrin and heme synthesis have been recognized. Not only are the reduced analogues of uro- and coproporphyrin intermediates, but there is also evidence of the participation of porphyrinogens with 3, 5, 6 and 7 COOH-groups (Granick and Mauzerall 1958 *a*, 1958 *b*). In disturbances of the protoporphyrin synthesis, porphyrin intermediates may accumulate in the erythrocytes. In one case of disturbed heme synthesis, Garby, Sjölin and Vahlquist (1957) found that the free erythrocyte coproporphyrin was increased. It is not improbable that other intermediates accumulate in the erythrocytes in other disturbances of the

[1] The difference between the results of the duplicate analyses appeared unrelated to the concentration of the porphyrin.

protoporphyrin synthesis. It is therefore desirable to identify the free porphyrins of the erythrocytes.

In the present method, the free erythrocyte porphyrins are extracted in two main fractions. The 5 *N* HCl fraction normally consists of protoporphyrin only and the 0.24 *N* HCl fraction is believed to contain coproporphyrin. However, since this separation is founded only on differences in the distribution coefficients, porphyrins with "HCl-numbers" close to or lower than that of coproporphyrin may be extracted in the 0.24 *N* HCl fractions together with coproporphyrin. Studies of the "HCl-number" for distribution between ethyl acetate and hydrochloric acid of porphyrins with 5, 6 and 7 COOH-groups (Chu and Chu 1959) have revealed that the "HCl-number" decreased when the number of COOH-groups increased. Thus all these porphyrins and uroporphyrin can be expected to appear in the 0.24 *N* HCl-extracts. The properties of the 3-COOH-porphyrin are essentially unknown, and it cannot be predicted in which fraction the main part of this porphyrin will appear.

Methods and experimental findings

5 N HCl extracts. In all analyses, the wave-length of the Soret band peak was noted and was always found at 409–410 mμ. A few analyses of the porphyrin in the extract were completed by paper chromatography according to Eriksen (1953). The individuals studied were normal adults, infants and cases of iron deficiency. In no case was a porphyrin other than protoporphyrin found[1]. A 3-COOH-porphyrin would probably not have escaped detection if the concentration were not too small since Nicholas and Rimington (1951) by paper chromatography were able to demonstrate the presence of a 3-COOH-porphyrin in the urine from a case of lead poisoning. Their method was very similar to Eriksen's method (1953).

[1] In some chromatograms, a fluorescent spot was seen just behind the solvent front. Since this spot occurred irregularly, it was considered to be an artifact.

TABLE 16. *Mean values of k in different groups.*

Group	Number of observations	*k*
Pure coproporphyrin	8	0.55
Erythrocytes from adults	10	0.53
Erythrocytes from newborns	10	0.58
Plasma from adults	10	0.54
Plasma from newborns	10	0.56
Erythrocytes from patients with iron deficiency	10	0.64

0.24 N Cl extracts. The distribution coefficient of the porphyrin in the 0.24 *N* HCl extracts has been studied in the following manner. Let *k* be the fraction of porphyrin that remains in the ethyl acetate after one extraction with 0.24 *N* HCl. If the amounts of a porphyrin in the consecutive extracts are $c_1, c_2, \ldots, c_n$, *k* equals c_2/c_1 or $c_n/c_{(n-1)}$. In the study, *k* could not be calculated by means of the porphyrin amounts in the three last extracts because in these the porphyrin concentration was too small. For the routine use, *k* was calculated from the expression $= \sqrt{c_3/c_1}$. Owing to errors in the fluorometric analyses, the *k* values will have, of course, a great random variation, but comparisons can be made between mean values of different groups.

Erythrocytes and plasma from ten normal adults and ten newborn babies were studied as well as erythrocytes from ten individuals with iron deficiency anemia. The mean values of k in these groups were compared to the mean value of k as obtained by analyzing eight samples of pure coproporphyrin III. As can be seen from Table 16, the mean values differed little from that of pure coproporphyrin; only in the last group (erythrocytes from sideropenic anemias) the mean k value was considerably higher. Attempts to study the porphyrin in the 0.24 N HCl extracts by paper chromatography were not successful.

Discussion

Earlier researchers have established that protoporphyrin is the main free porphyrin occurring in the human erythrocytes. Grotepass (1937) isolated the porphyrin and identified it by means of melting-point, and by spectral studies in different solvents, etc. Watson, Grinstein and Hawkinson (1944) made similar findings in erythrocytes from dogs and rabbits with stimulated erythropoiesis. The present findings confirm this. No traces of any 3-COOH-porphyrin have been found in erythrocytes from normal subjects or from patients with iron deficiency.

Watson (1950) noticed the presence of another porphyrin in erythrocytes from cases of pernicious anemia in remission; the porphyrin was extracted from ether by very dilute HCl. Schwartz and Wikoff (1952), studying mainly animals with stimulated erythropoiesis, isolated a corresponding porphyrin; its melting-point, spectrum and chromatographic behaviour indicated it to be identical with coproporphyrin. Sturgeon, Chen and Bergren (1958) report the chromatographic separation of the erythrocyte porphyrins. Coproporphyrin and uroporphyrin were found in the dilute acid extracts. However, the isolation of free uro- or coproporphyrin from normal erythrocytes has not been reported.

Chu and Chu (1959), studying copro-, uro- and 5, 6 and 7-COOH-porphyrins, found that, in general, an increase in the number of carboxyl groups was followed by a decrease of the "HCl-number" of an ethyl acetate/hydrochloric acid distribution. The 5-COOH-porphyrin was an exception, the HCl-number being the same as that of coproporphyrin.

In the present study, the "HCl-numbers" have been studied indirectly by observations of the porphyrin contents in the consecutive 0.24 N HCl-extracts. In all normal groups (plasma and erythrocytes from normal adults and from newborn babies) the observation suggested identity with coproporphyrin. This confirms the earlier observations, but it cannot be excluded that 5-COOH-porphyrin was present. Furthermore, small amounts of other porphyrins are probably not detected by this technique.

In patients with iron deficiency anemias, the observations suggested the presence of a porphyrin with higher HCl-number. Calculations revealed that this increase could not be explained by the admixture of protoporphyrin itself. However, in a series of ten determinations of pure protoporphyrin, the 0.24 N HCl extracts contained a fluorescing substance with still higher HCl-number. Thus the most probable explanation of the findings in iron

deficiency anemia is that the abundant protoporphyrin has formed degradation products which disturb the study of the 0.24 *N* HCl extracts.

4. Clinical application of the method

For the clinical use of the method, two additional questions had to be studied, viz. (1) is it necessary to wash the erythrocytes prior to the analysis and (2) must the leucocytes be removed?

Washing of the erythrocytes. In Chapter VIII, determinations of porphyrin in plasma are described. Protoporphyrin was found to be practically absent from plasma and thus the removal of plasma is of minor importance for the analysis of free erythrocyte protoporphyrin. In contrast, considerable amounts of a coproporphyrin-like pigment were present, corresponding to 0.4–3.2 μg coproporphyrin/100 ml plasma. It is therefore important to remove plasma before the analysis of ECP. After one centrifugation (1500 g, 30 minutes) with removal of supernatant plasma, the hematocrit of the sediment was 95 %. Considering the volume of "trapped plasma" of the hematocrit determination, the total amount of plasma left in the erythrocytes could be estimated to 10 % of the erythrocyte volume or less. Since the ratio between coproporphyrin-like pigments in plasma and free erythrocyte coproporphyrin generally is between 1:4 and 1:1, the omission of washing the erythrocytes can be calculated to increase the free erythrocyte coproporphyrin about 5 %. Considering the great random variation of the analysis and the risk of losses during the washings, this technique was thought justified.

Porphyrin in leucocytes. In order to exclude the possibility that important amounts of free porphyrins are present in the leucocytes, the following experiment was carried out. Whole blood from a normal male adult was centrifuged and the plasma and top cell layer removed. The remaining erythrocytes were resuspended in their own plasma but with the omission of the top cell layer. A leucocyte count revealed that 90 % of the leucocytes were removed by this procedure. After a second centrifugation, the concentration of free porphyrins in the erythrocytes was determined. The experiment was carried out in duplicate and the results were compared to that of two determinations performed on untreated samples of the same blood. The concentration of free copro- and protoporphyrin was essentially unchanged by the removal of the leucocytes.

Other methods used. Hematocrit determinations were made by a hematocrit centrifuge[1] after 5 minutes' centrifugation. During the early stages of the investigation, determinations were made by centrifuging 5 cm capillary tubes in an ordinary laboratory centrifuge at 1500 g during 30 minutes.

Hemoglobin determinations were made by the oxyhemoglobin or cyanmethemoglobin method, calibrated through the courtesy of the Department of Clinical Chemistry, University Hospital, Uppsala. Reticulocyte counts were performed after supravital staining during at least 20 minutes at room temperature using a 0.5 % saline solution of brilliant cresyl blue.

Standard statistical methods were em-

[1] International Micro Capillary Centrifuge, Model MB.

ployed. The significance of differences between mean values was judged by aid of the t-test.

5. Some general remarks on the method

Some inherent disadvantages and other characteristics of the method are pointed out below.

Because of the great random error, single determinations are able to reveal only rather considerable changes in the levels of the porphyrin. Thus, the statistical significance of a protoporphyrin difference smaller than 20 % or a coproporphyrin difference smaller than 40 % is low. This is true for porphyrin levels within normal limits; if the levels are increased, smaller percentual differences may be significant. On the other hand, differences found between subnormal values are almost always statistically insignificant. This is the case especially when coproporphyrin results are compared.

The isolation of the free protoporphyrin is far from quantitative. In this respect, the present method appears to be inferior to the method of Grinstein and Wintrobe (1948). The recovery of coproporphyrin is even less. Should corrections then be made for the low recoveries? It is generally assumed that the "free erythrocyte porphyrin" is actually free or present in some loosely bound form in contrast to the firmly combined protoporphyrin of the hemoglobin. Eriksen (1955) has found evidences of a protoporphyrin-globin complex. The protoporphyrin of this complex is included in the routine extraction and analysis of "free erythrocyte protoporphyrin". Possibly other porphyrin-protein complexes exist in the erythrocyte but to date no observation has been made supporting this. The corrected values obtained by the author's method are threfore supposed to represent all the non-heme (or -hemine) porphyrin in the erythrocytes. The magnitude of the corrections necessary to compensate for the losses during the analysis contribute to the inexactitude of the method. Comparisons between the results of different investigators cannot be made without due regard to the possibility of methodological differences.

In hematological investigations, studies of free erythrocyte proto- and coproporphyrin are not often necessary. In iron deficiency anemia, for instance (always accompanied by high levels of free erythrocyte protoporphyrin) the diagnosis is more easily made by aid of other laboratory tests. However, in cases which resembles the "sidero-achrestic" anemias (Heilmeyer 1959), studies of free porphyrins in the erythrocytes are important. In these cases and in other rare forms of hypochromic anemias, it would be of great interest to be able to identify the porphyrin, even in the isomeric form. In the present method, it is comparatively easy to identify protoporphyrin by its absorption peak in the Soret band; further evidence can be obtained by paper chromatography. In the present investigation, it has only been possible to characterize the free erythrocyte coproporphyrin by its distribution between ethyl acetate and dilute hydrochloric acid. The presence or absence of small amounts of porphyrins with 3, 5 or higher numbers of COOH-groups probably cannot be decided in this manner.

The hematoporphyrin which probably

may be formed from protoporphyrin during analysis disturbs the determination of coproporphyrin. When samples of erythrocytes with normal content of protoporphyrin are studied, the hematoporphyrin formation is negligible. But, when higher protoporphyrin levels are found, the results of coproporphyrin analyses must be judged cautiously.

The many steps of the analytical procedure are time-consuming. Attempts to shorten the procedure by reducing the number of extractions have not been successful. Reduction of the necessary volume of erythrocytes has also been considered, but this was found impossible since microcuvettes for the fluorometer were not available.

6. Comparison to earlier methods

In the present method, the recovery of added copro- and protoporphyrin is not quantitative. About 25 % of the coproporphyrin and 20 % of the protoporphyrin are lost during the analysis. Thus the results must be corrected. Higher recoveries can be obtained if a greater number of extractions are made. But this was found unsuitable for routine use. Also, the risk of losses, by the destruction of porphyrin, increases if the procedure becomes too time-consuming.

The correction values used for the lost porphyrins are only valid if the analyses are carried out exactly in the manner described. The volumes of the solvents and the number and time of extractions must always be the same. It is also important to avoid strong daylight in the laboratory. In the description of earlier procedures, it has for example been prescribed that extractions should be repeated until no red fluorescence is seen in ultraviolet light. However, in order to obtain constant recoveries in all determinations it must be safer to carry out a fixed number of extractions.

In earlier methods, corrections of the results have not been made for the losses of porphyrins. (Recovery experiments have been reported for the method of Grinstein and Watson 1948 only.) The present method should therefore give higher values than those obtained with earlier procedures.

The ratio between the protoporphyrin of hemoglobin and the free protoporphyrin in the erythrocyte is about 30,000:1. The heme moiety is split off from the hemoglobin during the first extraction and goes into the ethyl acetate/acetic acid mixture. Theoretically, it is not extracted from here by HCl. According to the author's experience, a contamination of the acid extract with hematin is inevitable. Even the final acid extracts seem to contain hematin. This absorbs light intensely in the 400 mμ-region. Thus the determination of protoporphyrin by its maximal light absorption in the Soret band will give erroneous results. Successful corrections can be made by a procedure suggested by Rimington and Sveinssen (1950) which includes measurements of the light absorption at 430 and 380 mμ. This method, adopted in the present investigation, has only recently been used by other authors (Rubino *et al.* 1958, Koskelo 1959, Prato *et al.* 1959).

The determination of coproporphyrin in erythrocytes is made fluorometrically and in the physical determination, no distinction can be made between copro- and

protoporphyrin. Therefore, to determine the coproporphyrin by itself, a separation of copro- and protoporphyrin by aid of their different solubilities is necessary prior to the fluorometrical determination. But the protoporphyrin concentration is much higher than the coproporphyrin concentration, and it is inevitable that traces of protoporphyrin are co-extracted in the coproporphyrin extracts. According to the method of Schwartz and Wikoff (1950), the protoporphyrin is removed by chloroform extraction. The present author has not found chloroform efficient. Instead, a procedure is used in which the amount of co-extracted protoporphyrin can be corrected for. The procedure includes six consecutive extractions with fluorometric determination of each extract. This may seem troublesome but two additional advantages are obtained.

Great amounts of porphyrin or admixture of hematin quench the fluorescence. If all extracts from one blood sample are combined, a quenching may not be observed and faulty results may be accepted. With the present method, quenching will always be recognized since in this case the regular decrease of fluorescence in the consecutive extracts does not occur. The second advantage is that the nature of the porphyrin is checked in the analysis. The measurements of the consecutive extracts allow a calculation of the porphyrin's distribution between ethyl acetate and hydrochloric acid. This is described further below.

Owing to the more efficient correction for the co-extracted protoporphyrin, the present method—other details being equal—should give lower coproporphyrin values than the method of Schwartz and Wikoff. However, the present method employs the more efficient extraction of 0.24 *N* HCl instead of 0.1 *N* HCl, which has the opposite effect.

The free protoporphyrin from normal human erythrocytes was identified by Grotepass (1937). He collected extracts from erythrocytes for three years and crystallized the protoporphyrin. Hitherto, the coproporphyrin has only been identified in extracts from erythrocytes of animals with stimulated erythropoiesis (Schwartz and Wikoff 1952).

In the present study, the protoporphyrin could be identified by the position of the Soret band peak and by paper chromatography. The presence of coproporphyrin in erythrocytes has gained support in this investigation by the distribution between ethyl acetate and dilute hydrochloric acid.

PART TWO

Clinical study

CHAPTER VI

Normal values in adults

The primary aim of the present investigation was to study the changes of ECP and EPP[1] during the first two years of extra-uterine life. For the following reason, it was considered advisable to study a number of normal adults as well. Methodological studies revealed that small variations in the analytical procedure may give considerable differences in the results. The findings of different authors are thus not necessarily comparable even if similar methods have been employed. It is equally uncertain if the present modification of the Schwartz and Wikoff (1952) method is comparable with the earlier methods. Thus basic values for adults are necessary for the evaluation of results obtained in infants and children.

Material. The series consisted of 20 males and 20 females, most of whom were doctors, students, nurses and other hospital and laboratory personnel. All were in apparently good health; no one had had infections during the preceding weeks. No one had donated blood during the preceding months. Most of the individuals were in the third or fourth decades of life. The blood samples were collected in the early afternoon and the subjects were not fasting. Hematocrit readings were not below 40 % in the males nor below 38 % in the females.

[1] *Abbreviations.* ECP = Free erythrocyte coproporphyrin. EPP = Free erythrocyte protoporphyrin. EP = Erythrocyte porphyrin (in earlier investigations, in which no difference has been made between copro- and protoporphyrin). The results are given in micrograms per 100 ml packed erythrocytes, abbreviated μg %.

Results

Free erythrocyte coproporphyrin.

The results are shown in Table 17. The results of earlier investigations are also shown in this table. As can be seen, the values of ECP of 20 normal adult males ranged from 0.7 to 2.3 μg %, the mean value was 1.3 μg %. The corresponding values in females were: range 0.3–2.3 μg %, mean 1.2 μg %. No significant difference was found between males and females. As can be seen from the diagram in Fig. 13, the distribution of the values of males and females was somewhat different. In males, no value below 0.7 μg % was found, but in females, six values were below 0.5 μg %. The relation between the time of taking the blood sample and the menstrual cycle was not recorded.

Free erythrocyte protoporphyrin.

The values for EPP in 20 normal adult males varied between 16 and 52 μg %. The mean was 30 μg %. The corresponding values in females were: range 18–51 μg %; mean 37 μg %. In the diagram (Fig.13)

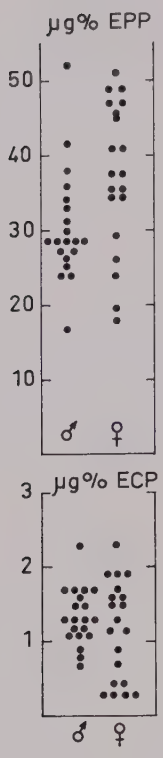

Fig. 13. Free erythrocyte copro- and protoporphyrin in healthy adults.

it may be observed that 19 of the males' values fell within 17 and 41 µg%. No abnormities were found in the individual with 52 µg% EPP, but studies of serum iron etc. were not performed. Hematocrit was 44%. The ECP was also high, 2.3 µg%. Duplicate determinations were made, the results of which were consistent.

The difference between the mean values of EPP found in males and females was found to be probably significant ($p < 0.05$).

Discussion

Free erythrocyte coproporphyrin. ECP values in normal adults have earlier been reported in four communications, the results of which are shown in Table 17. As can be seen, the present ECP results are similar to those found by Meduri, Notario and Casirola (1958). Prato, Mazza and Fiorina (1959), in a mixed material of males and females, found higher ECP, 0–3.5 µg%, on the average 1.5 µg%. The present results are higher than those found by Watson (1950) and by Krammer, Cartwright and Wintrobe (1954). In the studies

TABLE 17. *Present and earlier investigations of free copro- and protoporphyrin in healthy adults.*

Investigator	Year	No. of cases	ECP µg% Mean	ECP µg% S.D.	ECP µg% Range	EPP µg% Mean	EPP µg% S.D.	EPP µg% Range
Adult males								
Watson	1950	20	0.3	0.4	0 –1.0	29	4.5	24–42
Krammer *et al.*	1954	20	0.8	0.3	0.3–1.2	31	11.9	14–55
Meduri *et al.*	1958	15	1.1	0.7	0.3–2.5	30	10.7	18–49
Wranne	1960	20	1.3	0.4	0.7–2.3	30	7.5	16–52
Adult females								
Watson	1950	30	0.7	0.6	0 –2.0	42	5.9	32–52
Krammer *et al.*	1954	10	0.7	0.2	0.3–1.1	45	19.6	23–79
Meduri *et al.*	1958	15	1.3	0.6	0.5–2.8	31	8.1	21–47
Wranne	1960	20	1.2	0.7	0.3–2.3	37	10	18–51

of Watson and of Krammer *et al.*, the method described by Schwartz and Wikoff (1952) was used. It is believed, that the method used in the present investigation has given more efficient extraction and purification of the porphyrin. The higher coproporphyrin values now found are thus what could be expected from the methodological difference. In the report of Meduri *et al.*, the method for the determination of ECP is not clearly described. Prato *et al.* followed the just-mentioned method by Schwartz and Wikoff with two modifications: (1) plasma was not removed from the blood before analysis; (2) the final estimation of the coproporphyrin was made spectrophotometrically. According to the present author's findings, plasma of adults contains up to 1.5 μg % of a coproporphyrin-like porphyrin. Thus the high ECP results obtained by Prato *et al.* may be explained. It is also the present author's experience that spectrophotometry is inferior to fluorometry for the determination of ECP. Thus the relatively greater scattering of the ECP values found by Prato *et al.* may be explained.

Free erythrocyte protoporphyrin. The results of the determinations of EPP in males are almost identical in the investigations of Watson (1950), Krammer *et al.* (1954), Meduri *et al.* (1958) and in the present investigation (cf Table 17). In females, the results of these investigations are more varying. The average EPP values range from 31 to 45 μg %. The highest mean value was found in the study of Krammer *et al.* In their material, the range was remarkably great, the highest value found in one individual being 79 μg %. The possible causes of high EPP values will be discussed later.

The finding of similar mean values of EPP in the investigations made by aid of the Schwartz and Wikoff technique and by the author's method is remarkable. According to the methodological studies, the present modification of the Schwartz and Wikoff method is expected to give better extraction and hence higher EPP values. This should be particularly pronounced since corrections were made for the non-quantitative recovery of the present method. On the other hand, other differences in the methods may have been important; e.g., the present use of the correction by Rimington and Sveinssen which tends to decrease the results of EPP determinations.

Since the free erythrocyte coproporphyrin is small compared to the free erythrocyte protoporphyrin, comparisons can also be made with the earlier investigations of undifferentiated free porphyrin (Table 18). These investigations are in most cases performed according to the methods of Grinstein and Watson (1943) or Grinstein and Wintrobe (1948). These methods are essentially the same, the latter being a micromodification of the former. The determination of the protoporphyrin in the final extracts of the Grinstein and Watson method (1943) is generally performed with a photoelectric colorimeter, but some workers have made modifications: Ward and Mason (1950) use a fluorometer, Sturgeon (1954) a spectrophotometer. In the method by Grinstein and Wintrobe (1948) a spectrophotometer is always used. Here the recovery of added protoporphyrin was about 90 %. The results were not corrected for the 10 % loss. In most investigations, the results are calculated from the maximal light absorption

TABLE 18. *Earlier investigations of free erythrocyte porphyrin compared to the present results.*

Investigator	Year	Males			Females		
		Number of cases	Mean μg %	Range μg %	Number of cases	Mean μg %	Range μg %
Watson *et al.*	1944	8	26	20–36	4	38	31– 46
Cartwright *et al.*	1948	33	32	13–79	33	39	16–140
Bénard *et al.*	1949	8	18	9–27	4	18	14– 24
Ward and Mason	1950	40	44	13–92	37	42	17–139
Ventura and Klopper	1951				25	31	18– 50
Holly	1953				42	43	23– 67
Ventura and Meduri	1954	20	33	17–48	25	31	18– 50
Sturgeon	1954	34	23	10–50			
Sturgeon	1959				20	28	—[a]
Koskelo	1959	23	17	8–32	6	18	11– 21
Wranne[b]	1960	20	30	16–52	20	37	18– 51

[a] Selected material: only nulliparae. Range not given. S.D. ± 13 μg %.
[b] Free erythrocyte protoporphyrin.

in the Soret band. Only Koskelo (1959) uses the correction proposed by Rimington and Sveinssen (1950). In Table 18 the results of ten earlier investigations of EP are shown. As can be seen, the results are most varying, the mean EP values in male ranging between 17 and 44 μg %. The corresponding range of mean EP values of females is 18–43 μg %. The great variation between the results can be due to differences in the performance of the analytical procedure, but it may also be an effect of real difference between the groups of individuals studied. Iron deficiency and infections are known to increase the EP values (Cartwright *et al.* 1946, 1948) and the presence of infections or iron deficiency in a so-called normal material is perhaps conceivable. In the study of Holly (1953) the EP of 42 healthy, non-pregnant females was determined. The range was found to be 23–67 μg %. To four of these women, who showed EP values above 50 μg %, iron was administered. In each case, after treatment, the EP values was found to be below 50 μg % indicating a latent iron deficiency.

In the study by Cartwright, Huguley, Ashenbrucker, Fay and Wintrobe (1948) 33 normal males and 33 normal females were investigated. The mean EP in males was 32 μg %, and in females 39 μg %. The EP values ranged from 13 to 140 μg %. However, only three of the men and three of the women had EP values exceeding 60 μg %. It is tempting to postulate that some abnormal condition was present in these cases, but they are not separately discussed by these authors. In the study of Ward and Mason (1950), a few very high values were also recorded in the normal group, the highest being 139 μg %. These authors suggest that elevated EP values may be caused by mild chemical toxicity, in some cases including the use of alcohol.

On the other hand, two earlier investigations have given remarkably low values of EP, the mean value being below 20 μg %. In the investigation of Bénard, Gajdos and Canivet (1949), the number of in-

vestigated individuals is small: 8 males and 4 females. The statistical error of the mean is consequently great and the low mean values are perhaps explained by the random variation. Low values have also been reported by Koskelo (1959). These may be due to the fact that Koskelo used the Rimington–Sveinssen correction (p. 14), which often gives lower EP values.

The remaining reports of EP have given results which are in agreement with the present findings. Watson, Grinstein and Hawkinson (1944), Ventura and Klopper (1951), Ventura and Meduri (1954 a), Sturgeon (1954 and 1959) have in all investigated 62 males and 74 females. In males, the EP values range from 10 to 50 μg %, averaging 27 μg %. In females, the range is 18–50 μg %, averaging 31 μg %. These investigations were carried out by aid of the method of Grinstein and Watson (1943) or that of Grinstein and Wintrobe (1948). In the latter, the recovery of added protoporphyrin was about 90 % and it is probable that the method of Grinstein and Watson gives similar recovery. Thus the results should be multiplied by 1/0.9 in order to be comparable with the present results. After this correction, the mean EP in males is 30 μg % and the mean EP in females 34 μg %.

It seems justified to conclude that EPP determinations by the present method give results consistent with most of the earlier investigations of EP.

Individual variations of ECP and EPP.

Determinations of ECP and EPP were done at approximately the same time on different days during a period of two months in a healthy adult male. The ECP values were: 0.8, 0.8, 0.7, 0.7, 1.1 and 0.7 μg %. The EPP values were: 27.0, 35.4, 30.7, 26.6, 34.0 and 28.5 μg %. In another healthy adult male, determinations were made for a period of two years; i.e., during the major part of the time for this investigation. The ECP values were: 1.3, 1.1, 1.3, 1.3, 1.2 and 1.2 μg %. The EPP values were: 23.7, 29.5, 27.0, 28.7, 26.2 and 31.3 μg %.

The variations of ECP and EPP in the same individual appear to be smaller than those found between individuals. The finding of small variations of ECP and EPP within individuals is in accordance with earlier investigations. Cartwright *et al.* (1948), studying males as well as females, state that the variation of EP is not marked and that no correlation to the menstrual cycle can be observed. Ward and Mason (1950) found only small variations of EP. Ventura and Meduri (1954 a) could not establish any variations of EP during the course of the day, nor during the course of a year. Meduri *et al.* (1958), studying both EPP and ECP, found no significant variation during the menstrual cycle.

The present findings also indicate, that no gross systematic error occurred in the investigation.

CHAPTER VII

Normal values from birth to the age of two years

Material

Umbilical cord blood was obtained from twenty newborn babies delivered at the Obstetric Clinic in Uppsala. All deliveries were normal and occurred between 13 days before and 17 days after the expected time. There was no cases of asphyxia; birth weights ranged from 2500–4000 g; all mothers were healthy; hemoglobin levels of the mothers were not below 10 g/100 ml; there were no Rh-incompatibilities between mother and child and no child was abnormally icteric during the neonatal period.

Forty-one healthy infants and children from 9 days to 19 months old were also examined. Most of these were children nursed at the Pediatric Clinic in Uppsala for various reasons. A few were from two nurseries in Uppsala and Linköping. All infants were examined physically and care was taken to avoid individuals who were infected or had been infected during the previous weeks. However, it was inevitable, that the infants from the nurseries had had minor upper respiratory infections during the weeks prior to the investigation. These infants are therefore included in a special group. All infants and children had hemoglobin and hematocrit levels within the limits established by Albritton (1952). All samples consisted of venous blood taken in the fasting state between 7 and 9 o'clock in the morning. No distinction was made between boys and girls.

Results

The present results are shown and compared to earlier results in Tables 19 and 21. The individual values are shown in a diagram, Fig. 14.

Cord blood. ECP in cord blood varied between 1.1 and 7.2 μg % and EPP between 32 and 135 μg %. No correlation between the birth-weight or gestation time and porphyrin levels could be established. There was no distinct correlation between ECP and EPP variation, although the highest ECP value, 7.2 μg % was accompanied by the second highest EPP, 93 μg % and similarly the highest EPP, 135 μg % corresponded to the second highest ECP, 6.0 μg %. These children showed no abnormalities during the neonatal period. Labor had not been difficult. No anemia was present. They were physically normal at examination, and special hematological investigation was not performed.

The mean value of EPP was 54 μg % and of ECP 2.8 μg % and the standard deviations of the observations ± 25 μg % and ± 1.6 μg % respectively. If the two highest values of ECP and EPP are disregarded,

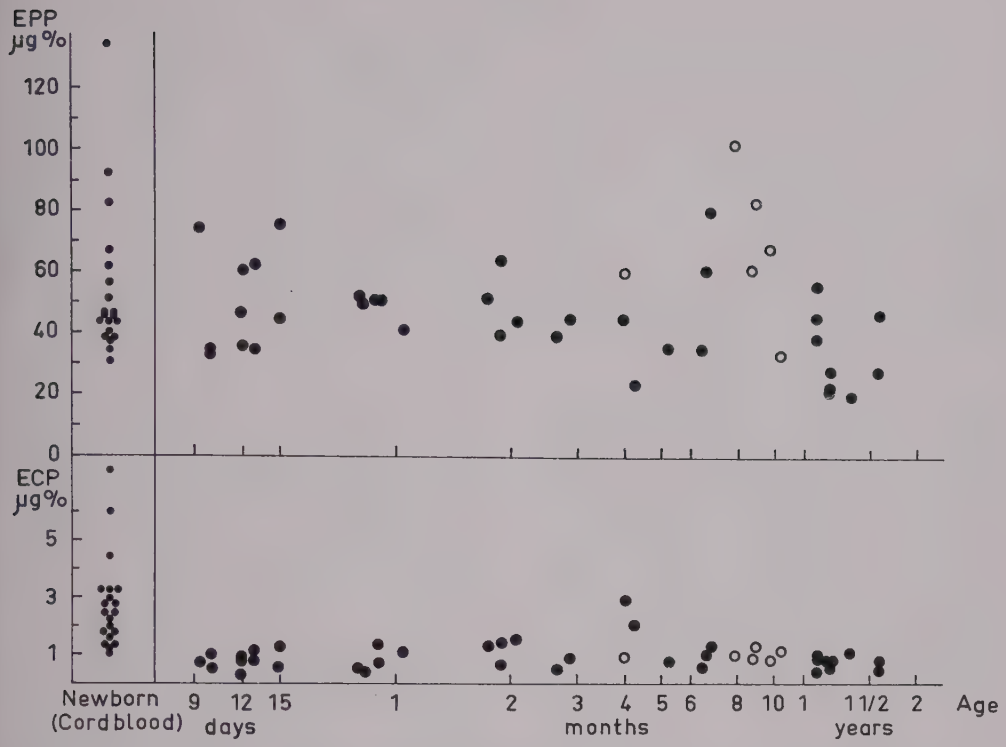

Fig. 14. Free erythrocyte copro- and protoporphyrin in healthy infants from birth to the age of two years. ○ = infants from nurseries.

the frequency distribution curves appeared to be approximately symmetrical. The medians were 48 and 2.5 µg %, respectively.

Infants and children 9 days–19 months old

The ECP level fell from a maximum level at birth to a very low level within the first two weeks. The difference between the level at birth and at the age of 9–15 days was statistically highly significant ($p < 0.001$). ECP levels remained normal or slightly low, compared to adults, during the rest of the investigated period. One ECP value was found astonishingly high, 2.9 µg % in a four month old girl, admitted to the hospital for a diaper rash of moderate intensity. However, no abnormalities could be found in the child and an error of analysis seems not improbable. Unfortunately, a reticulocyte count was not performed in this case.

The mean EPP of infants 9–15 days old was found to be 51 µg %, which is insignificantly lower than the mean value found in cord blood. In the age-group 15 days to 4 months, no certain trend towards lower values was found, the average being 49 µg %.[1] Infants 4 months to 12 months of age showed widely scattered EPP values, the lowest being 25 µg %, the highest 125 µg %. It should be noted that

[1] The mean EPP of infants 9 days–4 months old was significantly higher than the mean EPP of adult males as well as that of adult females ($p < 0.001$).

TABLE 19. *Free erythrocyte copro- and protoporphyrin in cord blood.*

Author	Number of cases	EPP, μg %		ECP, μg %	
		Mean	Range	Mean	Range
Schwartz and Wikoff 1952[a]	2	—	8.1; 5.3	—	178; 69
Hsia and Page 1954[a]	6	1.5	0.6– 2.8	125	63–203
Prato, Mazza and Fiorina 1959[b]	10	7.9	4.4–12.6	72	46–116
Wranne 1960	20	2.8	1.1–7.2	54	32–135

Methods: [a] Schwartz and Wikoff 1952; [b] Schwartz and Wikoff 1952, but coproporphyrin estimated spectrophotometrically. Plasma included.

among the four children having EPP values exceeding 70 μg %, three were inmates of nurseries.

The last group of children studied, aged 1–2 years, showed lower mean EPP level than the younger infants. The mean EPP of the former was found to be 32 μg %, only insignificantly higher than that of normal male adults.

Discussion

Comparison to earlier findings

Free erythrocyte coproporphyrin in cord blood has been studied by Schwartz and Wikoff (two cases, 1952), by Hsia and Page (six cases, 1954) and by Prato, Mazza and Fiorina (ten cases, 1959). The results of these investigations are compared to the present results in Table 19. Compared to the present findings, the two cases studied by Schwartz and Wikoff (1952) showed unusually high values. In contrast, the results of Hsia and Page gave lower mean ECP values than the present study. No normal material of adults were studied by these authors. One possible explanation of the divergent findings is that their method for ECP determination has given lower results than the present modification of the Schwartz and Wikoff method.

The method for ECP determination used by Prato, Mazza and Fiorina (1959) has been discussed earlier (p. 49). ECP values obtained by this method are probably too high owing to the inclusion of plasma in the analyses. However, their mean ECP value of cord blood is almost three times as high as the present. This difference cannot be sufficiently explained by the inclusion of plasma. The description of their technique is not detailed enough to allow a discussion of other causes of the difference.

Free erythrocyte protoporphyrin in cord blood has been studied by the authors just mentioned (Schwartz and Wikoff, 1952, Hsia and Page, 1954, Prato *et al.* 1959). Their results are also shown in Table 19. However, since the ECP is small compared to the EPP, comparison can also be made with the results of earlier investigations of EP (Table 20). As can be seen from the tables, the mean EPP (or EP) values found by the earlier authors are most varying. The range of the mean values found is 13 to 125 μg %. It is obvious that this great variation to a considerable degree must be due to methodological differences in the different investigations. In an attempt to compensate for methodological differences, the results of EPP (or EP) determinations

in cord blood were compared to the same author's results of determinations of erythrocytes from normal male adults. Such comparisons could be made in the investigations of Ventura and Meduri (1954a), Sturgeon (1954 and 1959), Prato, Mazza and Fiorina (1959) and in the present investigation. With the exception of Ventura and Meduri, all these investigators found that the mean EPP (or EP) of cord blood is about twice as high as that of normal male adults. In contrast, the study of Ventura and Meduri showed that in twelve newborn babies the mean EP was lower (29 μg %) than that of normal male adults (33 μg %). It has not been possible to find any explanation of this discrepancy. In the investigations in which the highest and lowest mean EPP (or EP) values were found, no adults have been included. In the study of Carletti (1956) the EP was studied in cord blood from thirty-two newborn babies. The range was found to be 3.4 –31 μg % and the mean value 12.8 μg %. It seems most probable that some profound difference in the performance of the determination has existed between the study of Carletti and other investigations. — The highest mean EPP value is reported by Hsia and Page (1954): 125 μg %. Six newborn babies were included. Since no adults were studied it is impossible to judge the significance of this result. Sturgeon (1954) has studied 41 infants, $\frac{1}{2}$–11 days old. The mean EP found in cord blood, 55 μg %, was found to be decreased about 10 μg % during the first two days of life. After that, it remained almost unchanged during the period investigated. This is in accordance with the present results. The age-group between $\frac{1}{2}$–4 months does not seem to have been investigated earlier. A great number of infants, 4 months to two years of age, have been investigated by Sturgeon (1954, 1958 and 1959). In the infants studied in 1954, mean EP values increased from the age of four months to a maximum of 160 μg % at the age of two years. The infants came from an underprivileged social group. Iron deficiency was probably common since, e.g., at twelve months' age the mean hemoglobin concentration was only 9.9 g/100 ml and the mean erythrocyte hemoglobin concentration 28.8 %. In another study, Sturgeon (1959) investigated infants and children from well-to-do families. As can be seen in Table 21 much lower EP values were found, essentially equal to the present findings. These infants were given a relatively advanced feeding routine, similar to that given to the infants in the present study. The effect of parenteral iron administration was also studied by Sturgeon (1958 and 1959). The injection of 250 mg iron at 9 months of age was followed by decreased EP values upon examination at 12 and 18 months' age.

TABLE 20. *Earlier investigations of free erythrocyte porphyrin in cord blood.*

Author	Number of cases	Mean μg %	S.D. μg %
Ventura and Meduri 1954[a]	12	29	9.7
Carletti 1956[a]	32	13	4.9
Sturgeon 1954[b]	22	55	16.9
Sturgeon 1959[b]	129	42	14.3

Methods: [a] Grinstein and Wintrobe 1948; [b] Grinstein and Watson 1943.

Physiological Aspects

During the first year of extra-uterine life, important hematological events occur. At birth, the hemoglobin content of blood

TABLE 21. *Present results of ECP and EPP determinations compared to the EP results of Sturgeon (1954 and 1959).*

	Sturgeon 1954		Sturgeon 1959			Wranne 1960		
Age group	No. of cases	EP µg %	No. of cases	EP µg %	Age group	No. of cases	EPP µg %	ECP µg %
Umbilical cord blood	22	55	129	42	Umbilical cord blood	20	54	2.8
2–11 days	31	45			9–15 days	10	51	0.8
					15 days–4 months	11	49	0.9
6 months		46	96	42	4 months–1 year	6[a]	48	1.5
12 months		104	91	35				
18 months		81	90	31	1–2 years	8	32	0.8

[a] Infants from nurseries excluded.

is high, serum iron elevated (Vahlquist 1941) and the total iron-binding capacity of serum slightly depressed (Smith *et al.* 1952, Hagberg 1953a). These findings as well as studies of the iron depots (Widdowson and Spray 1951) indicate that the depots are being built up. At birth, there are evidences of an accelerated erythropoiesis with reticulocytosis and extramedullary hematopoiesis. The high hemoglobin level also indicates that the erythropoiesis has been intense during the latter part of the fetal life. During the first few days of extra-uterine life, all signs of increased erythropoiesis disappear (Langley 1951, Seip 1955); during the following two months, decreased blood-formation and increased iron-storage occur (McCance and Widdowson 1951). During the remaining part of the first half-year, there is a successive return to full hematopoietic activity and simultaneously a successive decrease of serum iron and increase of the total iron binding capacity (Smith *et al.* 1952, Hagberg 1953a). These findings indicate that the iron depots are gradually emptied during this period. From the age of one or two years, normal adult conditions are gradually achieved.

The factors which cause the high hemoglobin level in newborn babies are not sufficiently known. Probably the most important factor is that the fetus *in utero* is living under a reduced oxygen tension. Studies of pO_2 by Sjöstedt, Rooth and Caligara in amniotic fluid (1958) and in the intervillous space of the placenta (1960) have indicated that pO_2 in the umbilical vein *in utero* is about 40 mm Hg and pO_2 in the tissues of the fetus probably is as low as 10 mm Hg. It thus seems justified to compare the newborn baby's high hemoglobin level with the polycythemia of high altitudes.

Can a relationship between the changes of free erythrocyte copro- and protoporphyrin from delivery to the age of two years and the physiological hematological processes just mentioned be found? Since it has been noted that several conditions with increased hemoglobin synthesis are followed by increased ECP (Watson 1950), the high values found in cord blood probably reflect the increased erythropoiesis. The rapid decrease in ECP, reaching normal or low levels by the second week of life, is also in accordance with the rapid disappearance of other signs of increased

erythropoiesis. However, other factors altering ECP cannot be excluded. The reduced oxygen tension of the fetal tissue may directly increase the ECP since studies of Falk, Porra, Brown, Moss and Larmine (1959) have established a direct effect of pO_2 variation on the biosynthesis of porphyrins and heme in chicken blood. The extramedullary erythropoiesis in the fetus may perhaps give erythrocytes with higher ECP content. Finally, the formation of hemoglobin F could be connected with high ECP level. Nevertheless, the rapid decrease of ECP after the delivery is not accompanied by a corresponding decrease in the formation of hemoglobin F. The formation of the latter has been found to continue during the first month of life (Vest 1959, Garby *et al.*, pers. commun.). After the first week of life, erythropoiesis seems to be depressed during the following two months. The low ECP values found are thus what could be expected. The ECP values remain normal or slightly low during the rest of the investigated period. This is in accord with observations that reticulocytosis does not normally occur during this age. It should be noted, however, that the significance of subnormal ECP values obtained by the present method is low.

The mean EPP in cord blood is doubled compared to the normal level for adult males according to the present investigations as well as to those of Sturgeon (1954 and 1959) and Prato, Mazza and Fiorina (1959). The most common causes of increased EPP levels, infections and iron deficiency, cannot have any influence in the newborn babies studied, since only healthy infants were included and since many investigations have established that no signs of iron-deficiency can be detected in newborn babies (for references, see above). The nutrition of the mother seems also to be of minor importance since in the study by Sturgeon (1959), the EP of the newborn child was not influenced by iron supplement to the pregnant mother. In the following, three additional causes of increased EPP will be discussed; viz., the accelerated erythropoiesis, the hypoxia of the fetus and the F-hemoglobin formation.

The accelerated erythropoiesis of the newborn baby may to some extent be the cause of the increased EPP in cord blood. In four cases of hemolytic anemia with reticulocytosis from 12–30 %, Watson (1950) found increased ECP, 30–50 μg %. The EPP levels, however, were only moderately increased—in three cases 60–65 μg %. Thus it seems doubtful that the increased erythropoiesis should be the only factor giving increased EPP in newborn babies.

The hypoxia of the fetus during intrauterine life may be an important factor. Interesting comparisons can be made with other conditions with hypoxia or impaired cardiopulmonary function. Six individuals living at high altitudes (15,000 feet) have been studied by Reynafarje, Lozano and Valdivieso (1959). The EP was found to be increased, averaging 105 μg %. The high EP could not be explained by other factors such as iron deficiency. After descent to sea level, the mean EP fell to 60 μg % after about two weeks and to about 55 μg % after one month.

The effect of short-time impairment of the pulmonary function has been studied by Bariéty, Gajdos, Török and Gimbert (1958). Air was insufflated in the mediastinum for diagnostic purposes in seven

patients with initially normal EP values. The insufflation was known to cause a transitory depression of pulmonary function as measured by the vital capacity, the maximum breathing capacity and the timed vital capacity (Bariéty, Coury and Gimbert 1958). After 72 hours, all tests were normalized. No clinical symptoms such as dyspnea or cyanosis occurred. An increase of EP levels was found during the week after the insufflation. The maximal values exceeded the original values by 58 to 206 %. On the eighth day, EP values were normalized. In three cases, hemoglobin concentration, red cell count and serum iron were also studied. No alterations were found. The authors conclude that the increase of EP was due to an increased synthesis of hemoglobin. The latter increase was thought to depend on the depressed respiratory function following insufflation. However, studies of ECP, reticulocytes or arterial pO_2 were not carried out.

In ten cases of congenital heart disease with secondary polycythemia, Koskelo (1959) found increased EP values in six of the cases. This was thought to be due to a "relative lack of iron available for the greatly increased hemoglobin synthesis".

These studies indicate that the EP levels are dependent on the tissue oxygen tension. It is not known whether the pO_2 affects the porphyrin and heme synthesis directly or if the increased EP is a secondary effect of an increased erythropoietic activity or a "relative lack of available iron". The studies of Falk *et al.* (1959) mentioned above indicate that a direct effect on the porphyrin synthesis is not improbable.

The formation of hemoglobin F could be connected with the increase of EPP. Studies of ten cases of thalassemia major (Sturgeon, Chen and Bergren 1958) did not, however, reveal any increase of EPP, nor did Watson's study (1946) of one case of this disease. Although these cases were not described in detail, it can be supposed that a considerable hemoglobin F formation occurred in many of them. Accordingly, hemoglobin F probably is of little or no importance for the actual changes of EPP.

During the first four months of life, the EPP has been found to be nearly constant, averaging 50 μg %. Compared to the mean EPP of normal adult males, this value is clearly elevated. Since iron deficiency seldom occurs before the age of four months and since the investigated infants had not been noticeably infected this EPP value is astonishing. In adults with conditions of increased EPP, decreasing values are usually seen within a few weeks or a month after the elimination of the causal factor. This was the case in the mountain inhabitants studied by Reynafarje *et al.* and in the patients investigated by Bariéty *et al.* However, in the latter study, it is uncertain how much of the change was due to ECP. The present author's study of iron deficient individuals treated with intravenous iron therapy revealed that EPP decrease generally was evident within a week.

Beginning at the age of three to four months and lasting up to one year, the EPP values have been found to be most varying. This is consistent with earlier findings. Since low EP values could be obtained by Sturgeon (1958 and 1959) by the parenteral administration of iron, the great variation seems to be an effect of the

well-known relationship between iron deficiency and increased EPP (or EP) values. However, an EPP-increasing effect of repeated infections cannot be excluded. In the present investigation, the relationship between the EPP and iron deficiency could not be studied.[1]

Children aged 1–2 years were found to have EPP levels only slightly higher than those found in normal adult males. This result is thought to indicate that no important iron deficiency was present among these children.

Children over 2 years of age were not studied since the investigations of children 2–15 years old made by Sturgeon (1954), Hsia and Page (1954), Ventura and Meduri (1954a) and by Carletti and Camerini (1958) have failed to establish any relationship between age and EPP. Moreover, repeated studies of iron metabolism have shown that no marked physiological changes occur before puberty (Vahlquist 1941, Smith *et al.* 1952, Hagberg 1953a and others).

[1] Owing to a technical mishap, all plasma samples intended for the determination of serum iron and iron binding capacity were lost during the analysis.

CHAPTER VIII

Porphyrin in plasma

The presence of porphyrin in plasma or serum has been much disputed for a long time. Schumm (1916 and 1939) never could find porphyrin in sera from normal individuals while Fischer and Zerweck (1924) reported the constant presence of a porphyrin similar to coproporphyrin. Van den Bergh, Grotepass and Revers (1932), studying more than 350 normal and pathological sera, found signs of porphyrin only in cases of obstructive jaundice and congenital porphyria. Similar findings were reported by Vigliani and Sano (1940). Vigliani and Angeleri (1936), Vigliani and Waldenström (1937) and Kench, Gillam and Lane (1942) found porphyrin, probably protoporphyrin, in serum from patients with lead poisoning. On the other hand, de Langen (1949) reports the presence of coproporphyrin in serum from plumbism patients. The latter author found traces of porphyrin, probably coproporphyrin, in normal sera also. Ward and Mason (1950), studying EP, also investigated porphyrin in the plasma. In five normal subjects, levels between 0.2–0.5 μg/100 ml were found, determined by fluorescence and calculated as protoporphyrin. In six cases of obstructive jaundice, higher values were found, up to 5.6 μg/100 ml; in cases of porphyria up to 8.4 μg/100 ml. No attempts to characterize the porphyrin were made. The constant finding of coproporphyrin in the urine of normal adults (Zieve, Hill, Schwartz and Watson, 1953) makes probable that coproporphyrin normally occurs in plasma.

Newborn babies and fetuses were studied by Fikentscher (1935), using a spectrographic technique. One to three μg/100 ml serum were found in the newborn contrasting to the absence of porphyrin in sera from the mothers. The spectrum of the plasma porphyrin closely resembled that of pure coproporphyrin I and III. The free erythrocyte proto- and coproporphyrin were not studied.

Results

Samples of plasma from twenty-seven normal individuals were examined. Sixteen were newborn babies and the blood was obtained from the cord. The rest were healthy adults. The samples were analyzed according to the author's method for determination of free erythrocyte copro- and protoporphyrin and, as a rule, 5 ml plasma was used. In some cases, however, only 3 or 4 ml were available. No significant amount of protoporphyrin-like pigment was found, but in the final 0.24 *N* HCl fractions a coproporphyrin-like pigment regularly appeared, although in small quantities. The distribution of this pigment between HCl and ethyl acetate

TABLE 22. *Plasma porphyrin in normal individuals.*[a]

	Number of cases	Mean μg/100 ml	Range μg/100 ml	S.D. μg/100 ml
Newborn babies	16	1.5	0.7–3.2	0.7
Adults	11	0.8	0.4–1.5	0.3

[a] The results are calculated as coproporphyrin.

was studied as described in Chapter V and found to be very similar to that of pure coproporphyrin and that of coproporphyrin from erythrocytes. Attempts at paper chromatography were not successful.

As can be seen from Table 22 and Fig. 15 the samples of plasma from adults contained 0.4–1.5 μg % of a coproporphyrin-like pigment. In newborns, the corresponding range was 0.7–3.2 μg %. In the figure, the individual values are presented together with the results of simultaneous analyses of free erythrocyte coproporphyrin. No significant correlation between plasma porphyrin and ECP could be found in the samples from adults or in the samples from newborn babies. In 22 of the 27 cases, the ratio between plasma porphyrin and erythrocyte coproporphyrin varied from 0.25:1 to 1:1.

In newborns, the mean plasma porphyrin was found to be 1.5 μg while in adults it was 0.8 μg/100 ml. The difference between the mean values found in normal adults and in newborn babies was found to be significant ($p = 0.01$).

In ten cases, double determinations were performed, and the error of the analysis calculated. The mean value of these determinations was 1.2 μg %, the standard error of a single determination was 0.25 μg %.

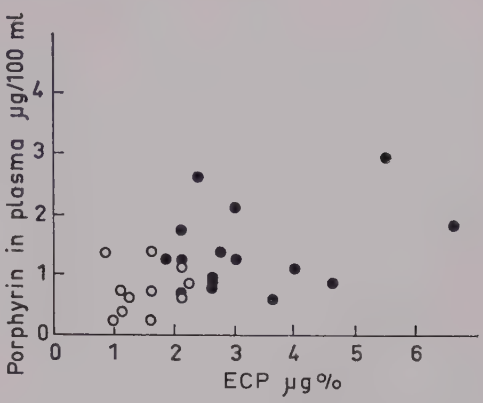

Fig. 15. Free erythrocyte coproporphyrin compared to porphyrin in plasma in adults (○) and in newborn infants (●). Plasma porphyrin calculated as coproporphyrin.

This result is in agreement with the corresponding error found in the analysis of free erythrocyte coproporphyrin.

Discussion

The nature of the plasma porphyrin. Before entering on a discussion of the identity of the plasma porphyrin, some attention must be directed to the question as to whether the observed substance really is a porphyrin at all. The presence of fluorescent substances in the final hydrochloric acid extracts after the numerous distributions between acid and organic solvents strongly indicate the presence of a porphyrin. It cannot be a product of impurities in the reagents as suitable corrections for blank values were employed (p. 33). Finally, the results of earlier investigators support the opinion that a real porphyrin exists in plasma.

In regard to the identity of the porphyrin in plasma, coproporphyrin seems to be the most probable; but this conclusion is founded only on studies of the distribu-

tion properties of the porphyrin. Thus hematoporphyrin could easily simulate coproporphyrin, but the former porphyrin has never been shown to occur normally in animals and its presence in plasma would seem, therefore, unlikely. The simultaneous presence of other porphyrins, similar to coproporphyrin, and probably present in the normal metabolism (e.g. the 3-COOH-porphyrin of Granick and Mauzerall, 1958b and 5-, 6- and 7-COOH-porphyrins, Granick and Mauzerall 1958a or even uroporphyrin) cannot be excluded. But in view of the present findings and the findings of earlier workers, it seems justified to assume that most of the plasma porphyrin is coproporphyrin.

The physiological importance of the plasma porphyrin is still unclear, since the origin as well as the elimination of plasma porphyrin are largely unknown. The presence of increased amounts of plasma coproporphyrin in a case of erythropoietic porphyria (Schmid, Schwartz and Watson 1954) indicates that some plasma porphyrin may derive from the hemoglobin synthesis. The probable enzymatic defect in this disease is an excess production of copro- and uroporphyrin of the I isomer, which, even normally, cannot be utilized for the synthesis of protoporphyrin IX and heme. In normal subjects, only small amounts of uro- and coproporphyrin type I are formed but are probably one source of the plasma porphyrin.

The free erythrocyte coproporphyrin may also contribute to the plasma porphyrin. Schwartz and Wikoff (1952), studying the isomeric composition of the free erythrocyte coproporphyrin in bled animals, found about 10 % type I and 90 % type III coproporphyrin. Since the former cannot be utilized and since coproporphyrin is most abundant in reticulocytes, there is a possibility that coproporphyrin I is passing out from the maturing erythrocytes into the plasma. All coproporphyrin type III may not necessarily be utilized for the synthesis of heme and protoporphyrin; it may also pass from the erythrocytes. A direct release of coproporphyrin from the bone marrow is also conceivable. Finally, the plasma porphyrin level is in some way related to liver function. In liver disease, increased amounts of porphyrin have been found by Ward and Mason (1950) and others, and porphyrin is also excreted in increased amounts in the urine (Watson, Capps, Rappaport and Hawkinson 1949). In the urine, the porphyrin was found chiefly to consist of coproporphyrin type I. The most probable explanation of these findings is that coproporphyrin normally is eliminated via the liver.

The findings of increased plasma prophyrin levels in newborn babies might be dependent on two factors: (1) the increased erythropoiesis during fetal life and at birth; (2) a deficient excretion by the liver. However, a certain elimination seems to occur since coproporphyrin has been found in meconium from newborn babies (Nicholas and Rimington 1951). According to Prato *et al.* (1959), the coproporphyrin is chiefly of type I.

CHAPTER IX

Changes of free erythrocyte copro- and protoporphyrin during iron treatment of iron deficiency anemia

The constantly high EPP of infants up to the age of four months has been found difficult to explain. In order to be able to compare with another condition with increased EPP, a number of patients with iron deficiency anemia were studied before and during intravenous iron treatment.

Studies of ECP and EPP during the specific treatment of iron deficiency anemia have not been reported earlier as far as can be ascertained. However, in three investigations, the changes of EP during iron therapy have been studied. Watson (1944) and Cartwright *et al.* (1946) followed the changes during oral iron administration; Pagliardi *et al.* (1959) studied the EP during intravenous iron therapy.

Material

Four women were studied. The diagnosis of iron deficiency anemia was based on the following hematological findings: anemia, low mean erythrocyte hemoglobin concentration, low serum iron, increased total iron-binding capacity of the serum. There were also "epithelial" symptoms in cases 1 and 2. A history of deficient nutrition was obtained in all cases but no. 4.[1] In Table 23, the hematological findings are summarized.

Two healthy women, 23 and 29 years old, were also studied. The criteria for their hematological normalcy were: hemoglobin concentration 13.0 and 13.4 g/100 ml, mean erythrocyte hemoglobin concentration 31.5 and 32.0 %, serum iron 171 and 114 μg/100 ml. No epithelial symptoms of iron deficiency were present. There was no history of increased menstrual blood loss.

Saccharated iron oxide containing 20 mg iron per ml was administered intravenously. At the first injection, 20–40 mg iron was generally given as a test dose. Since no untoward reactions occurred, the treatment was continued with injections of 100 mg iron every day or every other day. The total amount of iron given to the patients was 0.5–1.0 g. The healthy women were given 140 mg iron in all.

Results and Discussion

All patients responded to the iron administration by increased hemoglobin values. The average increase for a successful iron treatment established by Wintrobe (1956), a daily hemoglobin increase of

[1] In case 3, the iron deficiency was accompanied by pica, chiefly for match-tips. This habit was given up more than two weeks before the present study.

TABLE 23. *Initial hematological values in the patients.*

Case	Record no.[a]	Age	Hemoglobin g/100 ml	Hemato-crit %	Reticu-locytes %	MCHC %	S.I.[b] µg/100 ml	T.I.B.C.[b] µg/100 ml
1. E.H.	M 2340/59	16	6.8	27	2.4	24	43	392
2. H.E.	M 2593/59	76	7.8	28.5[c]	3.4	27	39	416
3. A.S.	P 184/60	14	6.6	26	3.0	25	8	413
4. M.S.M	O 391210	20	8.0	32[c]	—	25	30	435

[a] M: Medical Clinic, MO: Medical Clinic, out-patient department, P: Pediatric Clinic.
[b] Determinations of serum iron (S.I.) and total iron binding capacity (T.I.B.C.) were carried out in the Department of Clinical Chemistry of Uppsala.
[c] Venous blood.

0.17 g per 100 ml, was exceeded in all cases.

Initial values of EPP. The EPP was high in all patients before the therapy was started. The values found, 186–513 µg %, are consistent with those of Watson (1950) who found 180–390 µg %. Investigations of EP in sideropenic anemia has also been made by Watson (1946), his values varying from 50–600 µg %. Cartwright *et al.* (1948) found 100–475 µg %; Ventura *et al.* (1954b) 46–302 µg % and Pagliardi *et al.* (1959) 178–663 µg %. The observation of Watson (1946) that high EP values were more common in patients with "epithelial" symptoms seems to be supported, but the present material is too small to allow safe conclusions.

Changes of EPP during therapy. During the period of iron treatment, EPP was studied with short intervals. The results are shown in Figure 16–19. The day after the start of iron administration, blood samples were obtained from three patients. When compared to the initial EPP levels, two of the patients' EPP had increased; the other patient's EPP had decreased.

After ten to fourteen days, all patients had considerably lower EPP values than those initially observed. The most pronounced reduction was found in case 1: 215 µg %, corresponding to 42 % of the initial value. In cases 2, 3 and 4, the reduction was 84, 92 and 66 µg % respectively (28, 43 and 25 %). Cf Figure 16–19.

Since ECP was found to be small, comparisons can be made with earlier investigations. In three patients, Pagliardi *et al.* (1959) noticed that EP increased during the days after the start of therapy. In these patients, decrease of EP supervened after 3–10 days, but values below the initial levels were not achieved until the 7th to 22nd day.

In two other patients studied by Pagliardi *et al.*, the rate of EP reduction was even greater than that observed in the present study. One patient's EP decreased from 232 to 72 µg % in 10 days; another patient's EP was reduced from 179 to 82 µg % in 9 days. In the studies of Watson *et al.* (1944) and Cartwright *et al.* (1946) iron was administered orally. In Watson's patient EP decreased from 613 to 427 µg % in eight days. In Cartwright's three patients, EP decrease did not start until 10–20 days after the beginning of the therapy; after this start, the decrease of EP was rapid.

Initial values of ECP. The initial ECP

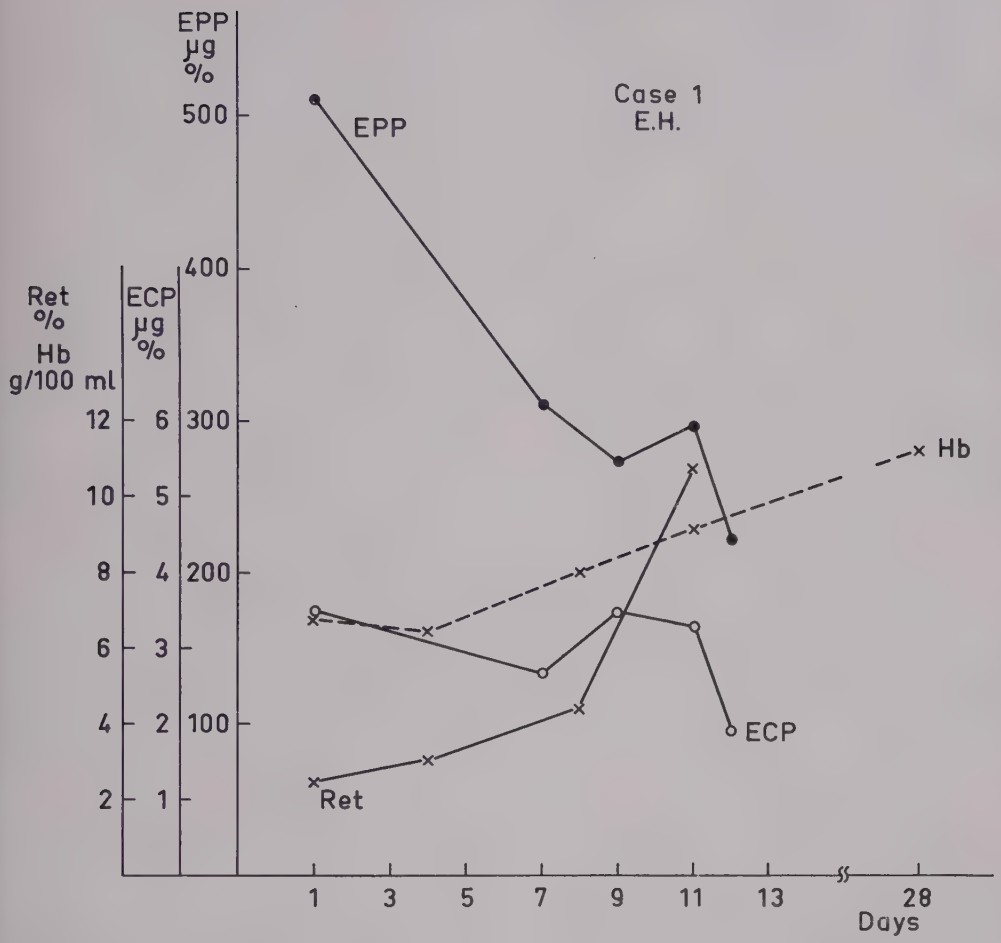

Fig. 16. Changes of free erythrocyte copro- and protoporphyrin in case 1, E.H. From day 1, iron was given intravenously. Hb = hemoglobin concentration. Ret = reticulocyte count.

values ranged from 0.9–3.5 µg %. In case 1, ECP was found to be 3.5 µg % which is higher than the maximum ECP found in normal females, but 2.4 % reticulocytes were also found.

Earlier, the ECP in iron deficiency anemia has been studied by Watson (1950). In four cases, ECP varied between 1.0 and 5.0 µg %. The highest value occurred in a patient with 4.2 % reticulocytes. The present findings confirm Watson's results.

Changes of ECP during therapy. Watson's study (1950) established a close correlation between ECP and the degree of reticulocytosis during the B_{12}-induced remission of pernicious anemia. The present investigation of iron deficiency anemia treated with iron did not reveal any correlation between ECP and reticulocyte count during the course of therapy (Fig. 16–18).

Results of ECP and EPP determinations in normal females. Initial values were within normal limits. After the intravenous

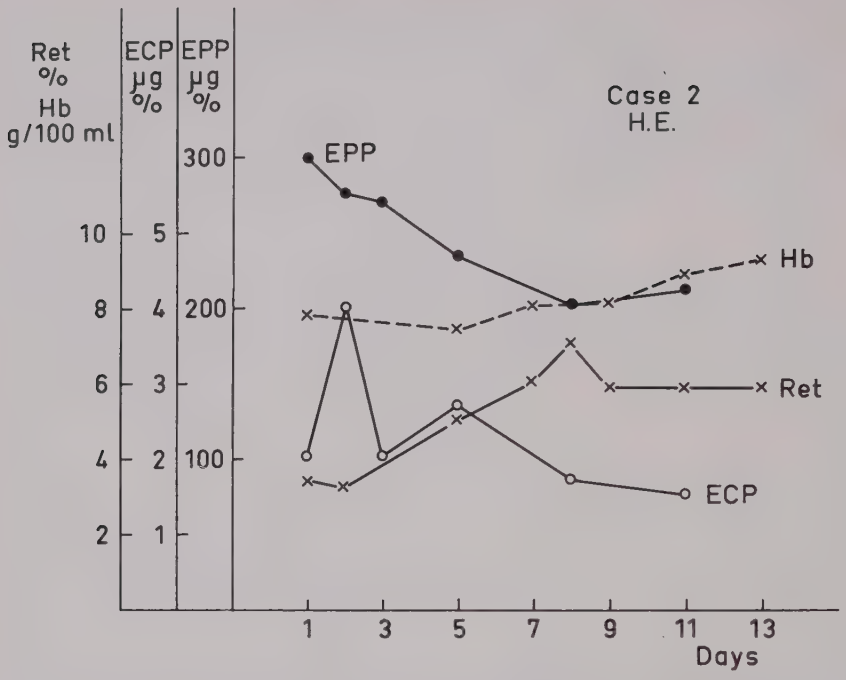

Fig. 17. Changes of free erythrocyte copro- and protoporphyrin in case 2, H.E. From day 1, iron was given intravenously. Hb = hemoglobin concentration. Ret = reticulocyte count.

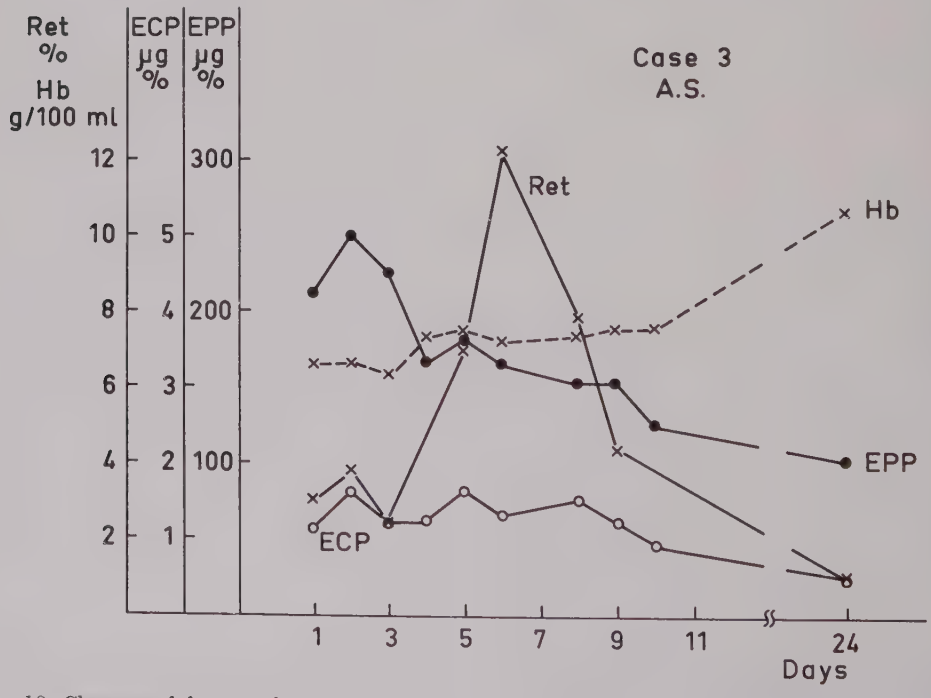

Fig. 18. Changes of free erythrocyte copro- and protoporphyrin in case 3, A.S. From day 1, iron was given intravenously. Hb = hemoglobin concentration. Ret = reticulocyte count.

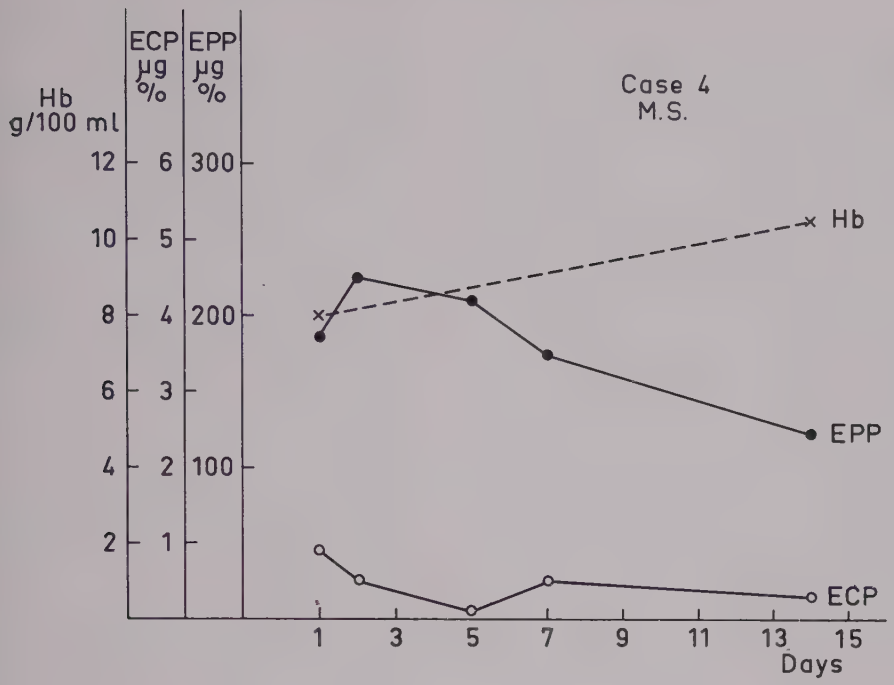

Fig. 19. Changes of free erythrocyte copro- and protoporphyrin in case 4, M.S. From day 1, iron was given intravenously. Hb = hemoglobin concentration.

administration of saccharated iron oxide, a slight decrease of EPP could be observed in one individual (Table 24). Simultaneously, the ECP was diminished in this individual. Hematocrit values remained unchanged.

Patho-physiological aspects

Protoporphyrin. Cartwright *et al.* (1948), discussing the EP of sideropenic anemia, have explained the increased EP in the following manner. In this anemia the

TABLE 24. *Changes of ECP and EPP after iron administration to healthy individuals.*

	Day	Iron given mg	EPP µg %	ECP µg %	Hematocrit %
L.B.	1	40	35.8	0.7	41.5
23 years	2	100	33.6	0.3	41.5
	6	—	27.5	0.1	41.5
	12	—	28.4	0.4	40
G.E.	1	40	40.9	0.5	42
29 years	2	100	40.2	0.5	42
	6	—	37.5	0.8	43
	12	—	39.5	0.4	43

porphyrin synthesis seems to be intact, while iron is not available for the synthesis of hemoglobin. Thus the free protoporphyrin is accumulated, representing an uncompleted hemoglobin synthesis. Since there were indications that reticulocytes contain more free protoporphyrins than mature cells (Watson and Clarke 1937; Grinstein *et al.* 1948), Cartwright *et al.* assumed that the free porphyrin in iron deficiency anemia was to be found chiefly in the reticulocytes. However, it was not explained how the free protoporphyrin disappeared during the maturing of the erythrocyte, whether by incorporation in hemoglobin, by catabolism or by removal from the erythrocytes.

Recent investigations by Goldberg (1959) and by Grinstein, Bannerman and Moore (1959) have established, that reticulocytes have the ability to form heme from iron and protoporphyrin. Mature erythrocytes do not seem to have the enzymes necessary for a noticeable heme synthesis. Thus it is assumed that the free protoporphyrin can disappear from the mature erythrocyte only by catabolism or by removal from the erythrocyte.

In the present study of EPP changes during iron therapy of iron deficiency anemia, it was found that during ten days the concentration of EPP decreased by 25–43 %. Similar and even greater EP-reduction has been found by Pagliardi *et al.* (1959). A catabolism or removal of the protoporphyrin caused by the injection of saccharated iron oxide *per se* seems unlikely, since the changes of EPP in normal controls were small. Assuming that (1) erythrocytes of all ages contain equal amounts of protoporphyrin, (2) the erythrocytes formed after the beginning of the iron therapy have very little protoporphyrin and (3) mature erythrocytes cannot synthesize heme from iron and protoporphyrin, the maximal reduction of EPP can be calculated from estimates of the total erythrocyte volume and of the rate of destruction of old erythrocytes. Using the hematocrit as an index of the changes of the total erythrocyte volume (Mollison 1956) and presuming a 10 % destruction of old erythrocytes after 10–11 days, such calculations have been carried out by the present author. It was found that in case 2 only, the rate of the EPP reduction was consistent with the three assumptions mentioned above. However, the finding of increasing EPP values during the first days following the beginning of iron therapy (cases 3 and 4) indicates that the newly formed erythrocytes contain considerable amounts of free protoporphyrin. If the observations just mentioned (Goldberg 1959 and Grinstein *et al.* 1959), are valid for mature erythrocytes of iron deficiency anemia, the conclusion must be drawn that the free protoporphyrin has not been distributed uniformly in the entire erythrocyte population before the treatment.

On the other hand, the free protoporphyrin is probably not present in the reticulocytes only. Observations failing to establish a relationship between the EPP and the reticulocyte count have been made by Watson (1944, 1946, 1950), and by others, in hemolytic anemia and during the treatment of pernicious anemia. Also, in the present study, no parallelism between the EPP concentration and the reticulocyte count could be found during the treatment.

Although the experimental findings do

not allow definite conclusions, the results suggest that the free protoporphyrin in iron deficiency anemia is present both in the reticulocytes and in the young mature erythrocytes. The mechanism by which the free protoporphyrin may disappear from the ageing erythrocyte is not known.

The immediate EPP-response to intravenous iron therapy was found to vary. In two patients, now studied, as well as in two patients observed by Pagliardi *et al.* (1959), the EPP increased during the first days of iron administration. In another case of the present study, the EPP at once showed a distinct downward trend. Stiefel and Jasiński (1954) have demonstrated that in animals the transition of labelled iron from saccharated iron oxide to the circulating transferrin is very rapid, labelled iron appearing in transferrin within an hour. After the injection of saccharated iron oxide in patients, an immediate transfer of iron to transferrin has been demonstrated by Hagberg (1953b). Thus an immediate decrease of EPP is possible although an increase is more often found. Further investigations are necessary for the explanation of this discrepancy.

The conclusions that can be drawn from the changes of ECP during iron administration to patients with iron deficiency anemia are limited by the uncertainty of ECP determinations in the presence of great concentrations of EPP (Chapter III). However, the present findings indicate that during the iron administration, no parallelism exists between the ECP level and the reticulocyte count. In this respect, the reticulocytes of iron deficiency anemia in remission differ from those of pernicious anemia in remission.

CHAPTER X

General discussion and conclusions

Since the detection of free erythrocyte protoporphyrin in 1928 by van den Bergh and Hyman, a comprehensive literature has accumulated on this subject. At least twenty publications have appeared on the quantitative determination of free protoporphyrin in erythrocytes. The determinations of the protoporphyrin content of normal *whole blood* have given contradictory results; in the study of van den Bergh, Grotepass and Revers (1932), 2–12 μg % were found, while in the study of Lageder (1936) a variation of 10–120 μg % was found.

Later, most authors calculated the protoporphyrin content per 100 ml *erythrocytes*, thereby enabling a comparison between normal and anemic subjects. These values, too, have been most varying. Schumm (1939) found the free erythrocyte protoporphyrin in normal subjects ranging from 11–17 μg %, with the average 13 μg %; while Ward and Mason (1950) found it ranging from 13 to 139 μg %, the mean erythrocyte concentration being 43 μg %. It seems unlikely that these widely scattered results are merely due to variations in the actual porphyrin content. According to the author's experience, a considerable variation in the results is obtained by small differences in the procedure.

Free erythrocyte coproporphyrin, first described by Watson in 1950, probably has been included in most of the earlier determinations of free protoporphyrin, and hence the name free porphyrin is more appropriate. In normal subjects, the concentration of free coproporphyrin is small compared to that of free protoporphyrin. The mean ECP values found in healthy adults range from 0.5 μg % (Watson 1950) to 1.5 μg % (Prato *et al.*, 1959). It seems likely that the variations in these values are due not only to real variations between the materials but also to differences in the analyses. It is, of course, important to remove the plasma from the erythrocytes since a coproporphyrin-like pigment has been found in plasma (Chapter VIII).

In the present investigation, our main attention has been directed to methodological work in order to make the method practical for routine clinical use. The different steps of the analysis have been investigated and a standardized procedure adopted. The influence of hematin on the protoporphyrin determination has been greatly reduced. The effect of co-extracted protoporphyrin on the coproporphyrin determination has been suppressed by a special procedure. Since recovery experiments revealed that losses of porphyrin during the analysis are inevitable, a special correction of the results was introduced.

The present investigation of normal adult males and females showed the mean EPP

to be 30 and 37 μg %, respectively. These findings are consistent with recent results. The mean ECP values found in males of 1.3 μg % and in females of 1.2 μg % are a little higher than those found by other investigators. This is thought to be due to differences in methods and/or correction procedures.

ECP and EPP were studied in cord blood from 20 newborn infants and in venous blood from 41 infants 9 days—19 months old (Chapter VII). At birth, both ECP and EPP were found to be elevated compared to the normal values of adults. The increased ECP in cord blood is assumed to be due to the increased erythropoiesis present at birth and during the last part of the fetal life. This assumption is supported by the finding of normal adult ECP values in full-term infants 9–15 days old. At this age, all signs of increased erythropoiesis generally have disappeared. The findings of increased EPP levels in cord blood are more difficult to explain. As has been concluded earlier (Chapter VII), it would seem probable that the most important factors causing the increased EPP at birth are (1) the hypoxia of the fetus, and (2) the accelerated hemoglobin synthesis. Of course, some special unknown conditions, occurring only in newborn babies, cannot be excluded. During the first four months of life, EPP values have been found to be essentially constant and distinctly increased when compared to normal adults. A perusal of the literature revealed that in other conditions with increased EPP, decreasing EPP values were found within a few weeks or a month after the elimination of the causal factor. Since the low oxygen tension of the fetus is abruptly alleviated by delivery and the accelerated rate of the erythropoiesis is changed into a normal or subnormal rate within the first week of extra-uterine life, the constantly increased EPP found in infants up to four months of age is left unexplained. Among other known factors causing increased EPP, e.g., iron deficiency, anemia of infection, lead poisoning, only iron deficiency could possibly occur in apparently healthy infants. However, iron deficiency is very seldom found before the age of four months.

It thus seems necessary to assume that the infant less than four months old deals with the free protoporphyrin in a manner different from that of the adult. It could be postulated that the infant's bone marrow, although subnormal in activity and adequately supplied with iron, is—for some reason—discharging red cells with an unusually high content of free protoporphyrin. But in that case, it is difficult to explain why the EPP is almost unaffected by the transition from the active erythropoiesis at birth to the depressed one of full-term infants one week to three months of age.

It could also be suggested that some physiological minimum level of EPP exists. This level, then, could be higher in infants than in adults. In fact the earlier and present investigations of normal adults have revealed that EPP (or EP) values seldom are lower than 15–20 μg %. Even in patients with aplastic anemia and biochemical signs of well-filled iron stores and in patients with hemochromatosis, the EPP values are not much lower (Ventura *et al.* 1954 b). Thus a minimum level of EPP seems to occur in adult individuals. A similar phenomenon in very young infants does not appear improbable.

Yet it is difficult to suggest a mechanism by which the varying "minimum EPP level" is caused. One possibility may be that the free protoporphyrin is not a uniform substance. Considering coproporphyrin, at least three different analogues can be expected to occur in the erythrocytes; viz., coproporphyrinogen III, an intermediate of heme synthesis; coproporphyrin III, the oxidized analogue of the former; coproporphyrin I. Only coproporphyrinogen III can be utilized for the synthesis of protoporphyrin and it is probable that this process also takes place in circulating reticulocytes. It may be, therefore, that the coproporphyrinogen III concentration in the reticulocytes decreases while that of coproporphyrin I and III remains unchanged. Thus a "minimum ECP level", caused mainly by the two latter porphyrins could be explained.

Protoporphyrin IX, on the other hand, is the precursor of heme (Heikel, Lockwood and Rimington 1958). The reduced analogue, protoporphyrinogen, is probably easily autoxidized. No isomers other then type IX have been found. Thus for protoporphyrin, a "minimum EPP level" cannot be explained in this manner.

Studies of iron deficiency anemia have been described in Chapter IX. It was concluded that the increased EPP was situated chiefly in reticulocytes and young mature erythrocytes in these patients. Thus a process must be assumed by which the EPP content is reduced during the ageing of the erythrocytes. The rate of this reduction can be expected to influence the minimum EPP level. If this rate is slower in the very young infant than in the adult, an increased minimum level of EPP would follow.

The present investigation was undertaken to study the physiological behaviour of free copro- and protoporphyrin during infancy. Characteristic changes of the free porphyrins in disease have been noted by earlier investigators in, among others, the anemias of hemolysis, iron deficiency and infection. Furthermore, a disturbance of the porphyrin metabolism with altered levels of free porphyrins has been found not only in such diseases as erythropoietic porphyria but also in a certain type of hypochromic anemia (Garby, Sjölin and Vahlquist 1957). Additional cases have been reported recently. Determinations of free erythrocyte copro- and protoporphyrin can, therefore, contribute to the diagnosis of cases of atypical hypochromic anemia.

Summary

Methodological Studies

A method for the simultaneous determination of free erythrocyte copro- and protoporphyrin is described. Coproporphyrin is determined fluorometrically after a final extraction with 0.24 *N* HCl, while protoporphyrin is determined spectrophotometrically after a final extraction with 5 *N* HCl. The details of the analysis have been investigated and recovery experiments performed. Since losses of porphyrins always occurred, suitable corrective equations were used.

The nature of the extracted porphyrins was checked. Protoporphyrin was identified in the 5 *N* HCl extracts. In the 0. 24 *N* HCl extracts, the identity of coproporphyrin could be experimentally corroborated.

Clinical Studies

In healthy male adults, the mean concentration of free erythrocyte protoporphyrin was found to be 30 μg % and that of coproporphyrin 1.3 μg %. The corresponding mean values in females were 37 μg % and 1.2 μg %.

Divergent values were found in early infancy. In cord blood, the content of free protoporphyrin was 54 μg % and that of coproporphyrin 2.8 μg %. During the first week of life, the latter concentration decreased rapidly; in infants 1–2 weeks old, the mean concentration was down to 0.9 μg %. The free protoporphyrin remained nearly unchanged during the first four months of life. After that, fluctuating values supervened. Beginning about the age of one year, the values approached those for normal adults.

The free protoporphyrin has also been studied in erythrocytes from iron deficiency patients before and during iron therapy. The protoporphyrin concentration decreased considerably during treatment.

In plasma, a coproporphyrin-like pigment has been found. The mean concentration in adult plasma corresponded to 0.8 μg coproporphyrin per 100 ml. In cord blood plasma, the concentration corresponded to 1.5 μg per 100 ml.

The findings are discussed and put in relation with other known physiological processes during infancy. The conclusion is drawn that erythrocytes of the very young infant deal with the free protoporphyrin in a dissimilar manner from that of the adult's erythrocytes.

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